AD_____

GRANT NUMBER DAMD17-94-J-4157

TITLE: A Novel Model System to Examine Agents Used in Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: Jennifer M. Coll

CONTRACTING ORGANIZATION: University of Maryland at Baltimore
Baltimore, Maryland 21201

REPORT DATE: July 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Final (1 Jul 94 - 30 Jun 98)

4. TITLE AND SUBTITLE
A Novel Model System to Examine Agents Used in Breast Cancer Therapy

5. FUNDING NUMBERS
DAMD17-94-J-4157

6. AUTHOR(S)
Jennifer Coll

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland at Baltimore
Baltimore, Maryland 21201

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)* We have isolated a multiprotein complex for DNA synthesis, designated the DNA synthesome, from human breast cancer (MDA MB-468) cells, biopsied human breast tumor tissue and xenografts from nude mice injected with the human breast cancer cell line MCF-7. The breast cell DNA synthesome was shown to fully support the *in vitro* replication of simian virus 40 (SV40) origin-containing DNA in the presence of the viral large T-antigen. The proteins and enzymes found to copurify with the breast cell DNA synthesome include: DNA polymerases α, δ, and ε, DNA primase, proliferating cell nuclear antigen (PCNA), replication factor C (RF-C), replication protein A (RP-A), DNA ligase, DNA topoisomerases I and II and poly(ADP-ribose) polymerase. To begin to determine the organization of these DNA synthetic proteins within the breast cell DNA synthesome, we performed co-immunoprecipitation experiments with antibodies directed against DNA polymerases α, δ and PCNA. We found that DNA polymerases α, δ, DNA primase, RF-C and PCNA tightly associate with each other in the complex, whereas DNA polymerase ε, PARP and several other components interact with the synthesome via an association with only PCNA or DNA polymerase α. Furthermore, we employed the breast cell DNA synthesome as a model to study the mechanisms of action of two anti-breast cancer agents that target the DNA synthetic process, irinotecan (CPT-11/SN-38) and etoposide (VP-16). We obtained novel data suggesting that both SN-38 and VP-16 stabilized cleavable complexes represent blocks to replication fork progression, as each agent caused an accumulation of short DNA products during synthesome mediated *in vitro* replication. Overall, our results indicate that breast cancer cells utilize an asymmetric multiprotein complex to mediate DNA synthesis and that utilization of the DNA synthesome as a drug model may provide important new insights into the mechanisms of action of SN-38 and VP-16.

14. SUBJECT TERMS
Breast Cancer
Multiprotein Complex; DNA Replication; Irinotecan; Etoposide

15. NUMBER OF PAGES
194

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

JC Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

JC Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

JC In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

JC In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

JC In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Jennifer M. Call 8/10/98

PI - Signature Date

Abstract

The DNA Synthesome: A Model for Studying Breast Cancer Cell DNA Replication and the Mechanisms of Action of Anti-Breast Cancer Agents

Jennifer M. Coll, Doctor of Philosophy, 1998

Dissertation directed by: Linda H. Malkas, Ph.D., Associate Professor, Department of Pharmacology and Experimental Therapeutics

We have isolated a multiprotein complex for DNA synthesis, designated the DNA synthesome, from human breast cancer (MDA MB-468) cells, biopsied human breast tumor tissue and xenografts from nude mice injected with the human breast cancer cell line MCF-7. The breast cell DNA synthesome was shown to fully support the *in vitro* replication of simian virus 40 (SV40) origin-containing DNA in the presence of the viral large T-antigen. Moreover, our results obtained from a forward mutagenesis assay indicate that the DNA synthesome isolated from malignant breast cells possesses a lower fidelity for DNA replication *in vitro* than the complex from a nonmalignant breast cell line. The proteins and enzymes found to copurify with the breast cell DNA synthesome include: DNA polymerases α, δ, and ε, DNA primase, proliferating cell nuclear antigen (PCNA), replication factor C (RF-C), replication protein A (RP-A), DNA ligase, DNA topoisomerases I and II and poly(ADP-ribose) polymerase. To begin to determine the organization of these DNA synthetic proteins within the breast cell DNA synthesome, we performed co-immunoprecipitation experiments with antibodies directed against DNA polymerases α, δ and PCNA. We found that DNA polymerases α, δ, DNA primase, RF-C and PCNA tightly associate with each other in the complex, whereas DNA polymerase ε, PARP and several other components interact with the synthesome via an interaction

with only PCNA or DNA polymerase α. Furthermore, we employed the breast cell DNA synthesome as a model to study the mechanisms of action of two anti-breast cancer agents that target the DNA synthetic process, irinotecan (CPT-11/SN-38) and etoposide (VP-16). We obtained novel data suggesting that both SN-38 and VP-16 stabilized cleavable complexes represent blocks to replication fork progression, as each agent caused an accumulation of short DNA products during synthesome mediated *in vitro* replication. Overall, our results indicate that breast cancer cells utilize an asymmetric multiprotein complex to mediate DNA synthesis and that utilization of the DNA synthesome as a drug model may provide important new insights into the mechanisms of action of SN-38 and VP-16.

Table of Contents

List of Figures

CHAPTER.....PAGE

List of Tables

Chapter 1: Introduction

I. BREAST CANCER

Breast cancer is the most commonly diagnosed female cancer and the second leading cause of cancer death among women in the United States [1]. The incidence of breast cancer occurrence has increased steadily over the past several decades in almost all developing countries and it is estimated that 25% of patients diagnosed with node-negative breast cancer will eventually develop recurrent tumors and are at risk of death [2]. Risk factors for the development of breast cancer include both personal and environmental elements: family history of cancer, higher socioeconomic class, first childbirth after age twenty-five, early age of menarche, late age of menopause, a high dietary fat intake and exposure to ionizing radiation [2,3]. Currently, intense research efforts are underway to understand the molecular mechanisms of breast cancer progression and develop improved approaches for the detection and treatment of breast cancer.

According to one model for breast cancer progression, the disease can be divided into a series of morphologically distinguishable stages, beginning with benign hyperplasia, which develops into atypical hyperplasia, then *in situ* carcinoma and finally invasive cancer [4]. Evidence for a continuum between proliferative breast disorders and breast cancer stems from several clinical observations. For example, atypical hyperplasia of breast cells is associated with a ten-fold increased risk for the development of breast carcinoma when found in women with a positive family history for breast cancer [5]. Additionally, a majority of infiltrating ductal carcinomas contain *in situ* components, the proportion of which varies from 10%-90% [6,7].

Despite some compelling support for the morphological continuum model for breast cancer progression, many invasive cancers do not contain atypical components. This observation suggests that these cancers may have originated directly from morphologically normal breast tissue. As further evidence against the continuum model, no stepwise acquisition of genetic events is associated with the transition from atypical hyperplasia to invasive breast cancer [8].

In fact, at the molecular level, breast cancer evolves by the clonal expansion of cells that have acquired various genetic aberrations that provide them with selective growth advantages: deregulated proliferation, tissue invasiveness, angiogenesis and the ability to metastasize [9]. A widely accepted theory for breast cancer progression, the stochastic model, maintains that each phenotype required for malignancy can be accomplished by several genetic lesions, with some lesions causing a more aggressive phenotype than others (Figure 1) [8]. Additionally, the phenotypic changes required for the development of invasive cancer may be acquired by tumor cells in any order. For example, when cells acquire the ability to invade basement membrane before the potential for deregulated growth, the invasive tumor will not proceed through a morphologically distinguishable *in situ* stage. The stochastic model for breast cancer progression is substantiated by comparative analyses of genetic alterations between pre-invasive and invasive breast tumors, where amplification of *c-myc*, abnormal expression of *RB-1* or loss of heterozygosity (LOH) at chromosome 17p each correlates with deregulated proliferation [10-12]. These studies also reveal that a number of LOH lesions, particularly at 11q23 and 11q13, may appear either early or late in breast cancer

Phenotypic Change:	Dysregulated Growth	Invasion	Angiogenesis	Growth at Metastatic Site
Increasing Aggressiveness ↓	D-1	I-1	A-1	G-1
↓	D-2	I-2	A-2	G-2
↓	D-3	I-3	A-3	G-3
↓	D-4	I-4	A-4	G-4
	$4^4 = 256$ types of breast cancer			

Figure 1. Stochastic model of breast cancer progression. This model accounts for the extreme biological heterogeneity among breast cancers. Numbers 1-4 in each column represent a hypothetical set of 4 genes whose aberrant expression allows the acquisition of the 4 malignant phenotypes indicated. Thus a large variety of combinations (256 in this example) are possible. During malignant progression, these genetic lesions are acquired stochastically. Depending on which gene/genes in the set becomes abnormal, the resulting tumor can be more or less aggressive in attaining the indicated phenotype.[8]

progression [13-16]. Moreover, the stochastic model accounts for the often markedly different clinical outcomes between cancers of similar stage and histology.

Among the genetic aberrations associated with carcinoma of the breast, mutations in the *p53* tumor suppressor gene are the most frequently identified lesions [17]. p53 is a 393 amino-acid nuclear phosphoprotein transcription factor that acts as a guardian of the genome by triggering G_1 cell cycle arrest or apoptosis in response to DNA damage [3;18,19]. Such a role for p53 is supported by studies demonstrating that ionizing radiation induces G_1 cell cycle arrest in p53 negative cells after their transfection with wild-type *p53* constructs and that p53 is essential for the initiation of apoptosis in murine cortical thymocytes after their treatment with the anticancer agent, etoposide [20,21]. The transient delay mediated by p53 at the G_1/S boundary permits the repair of damaged DNA prior to the onset of DNA replication. In the event that repair mechanisms fail, p53 triggers apoptosis, thus preventing the propagation of cells that have sustained mutations. Alterations of the *p53* tumor suppressor gene abrogate the genome-fidelity-monitoring function of normal p53 and, consequently, lead to the accumulation of a number of mutations throughout the genome. Such mutations include LOH at chromosomes 3p and 7q, amplification of the *c-erbB2* gene and allelic loss on chromosome 17 [22,23]. Furthermore, point mutations in the *p53* gene generate mutant polypeptides with an increased half-life, allowing their immunohistochemical detection under conditions where cells expressing wild-type p53 do not stain [24]. Several immunohistochemical studies have shown a correlation between p53 immunopositivity of breast tumor cells and a high S-phase fraction [25]; as well as high tumor histologic grade, overexpression of the

epidermal growth factor receptor (EGF-R) [26-28] and poor prognosis for patient survival [27].

In addition to *p53* tumor suppressor gene mutations, other genetic aberrations frequently identified in sporadic breast cancer include: overexpression of the EGF-R as well as the c-erbB2 and c-erbB3 type 1 growth factor receptors; amplification of *c-myc* and *cyclin D1*; and LOH at the sites of the *RB-1*, *BRCA2*, *maspin*, *nm23-1* and *nm23-2* tumor suppressor genes [8]. As with *p53* gene mutations, each of these genetic alterations (excluding LOH at *maspin, nm23-1* and *nm23-2* loci) has been shown to correlate with a high tumor proliferation index. For instance, elevated expression of EGF-R, identified by one group in 46/99 primary breast tumors (stage I-IIB), was found to associate closely with both a high S-phase fraction and poor patient prognosis [29]. Additionally, several investigators have determined that the overexpression of c-erbB2 and the amplification of *c-myc* in primary mammary carcinomas each correlates with a high proliferation index and unfavorable clinical outcome [10;30,31]. Finally, there is a substantial body of evidence that abnormal expression of pRb, identified in a large number of primary breast tumors, is related to both a high S-phase fraction and high mitotic index [11;32]. These data demonstrate that many different pathways can lead to deregulated cellular proliferation during the transformation process: upregulated growth factor receptor expression (EGFR, c-erbB2); increased expression of transcription factors that serve as positive modulators of cell growth (Myc); or abrogation of tumor suppressor function (p53, pRb). Furthermore, these results underscore the importance of the high proliferation phenotype to aggressive breast cancer progression.

Indeed, the proliferative activity of breast cancer cells has recently been put forward as an important prognostic factor, particularly when measured by DNA flow cytometry or ^{3}H-thymidine label incorporation into DNA, as compared to other methods [33-35]. Overall, tumor cell kinetics can independently predict metastatic potential, relapse-free and overall patient survival as well as response of tumors to chemotherapy and hormonal therapy [33,34;36-38]. Data from one clinical research study performed by McGuire et al. demonstrate that a higher percentage of node-negative breast cancer patients with tumors (diploid) containing a low S-phase fraction enjoyed a longer disease-free survival than those with tumors (diploid) and a high S-phase fraction [33;39]. It is hypothesized that an elevated proliferation index represents such an important prognostic factor in primary breast cancer because, first, it may signal that as yet unidentified genes associated with metastasis have been turned on or genes that suppress invasion or metastasis have been turned off [40]. For example, as mentioned earlier, it has been demonstrated that elevated proliferation rates correlate with overexpression of c-erbB2 and/or mutations in the *p53* tumor suppressor gene [25;30]. Such associations may partly explain why a high S-phase fraction predicts early disease recurrence in breast cancer patients. Second, a simpler explanation for the close relationship between a high tumor proliferation rate and poor patient prognosis is that fast-growing tumors proceed more rapidly through the stages of breast cancer progression than slow-growing tumors [40].

In addition to a high proliferation phenotype, it has recently been shown that mammary cancer cells possess a significantly lower fidelity for DNA replication than non-malignant breast cells [41]. Such a decreased fidelity for DNA synthesis suggests that transformation alters the process by which the DNA replication machinery of breast cells

replicates and/or participates in the repair of DNA. One explanation for the low fidelity for DNA replication of breast cancer cells stems from the observation that specific DNA synthetic proteins are targets for molecular modification during cellular transformation [42]. DNA polymerases α and ε purified from Novikoff hepatoma cells, for instance, have altered physicochemical and catalytic properties compared to the respective polymerases isolated from normal liver cells [42]. During DNA synthesis, these altered molecular and catalytic properties may contribute to a decreased specificity for nucleotide selection by the polymerases, which in turn leads to an increased mutation rate and, in part, overall genomic instability. Despite the importance of the DNA synthetic process to breast cancer progression, there is a paucity of information regarding the precise molecular mechanisms and regulation of human breast cell DNA replication.

II. SV40 DNA REPLICATION *IN VITRO*

The advent of cell-free based DNA replication systems, in particular that of the simian virus 40 (SV40) [43,44], has greatly facilitated the study of semi-conservative mammalian DNA synthesis *in vitro* [45]. SV40 is a small DNA tumor virus and a member of the papovavirus family [46]. The SV40 genome consists of a 5,243 base pair, double-stranded, covalently closed circular chromosome, the replication of which occurs in the nuclei of both permissive monkey and human cells. Viral DNA replication proceeds bidirectionally and semi-conservatively from unique SV40 origin sequences, terminating approximately half-way around the molecule from the point of initiation. The pre-initiation reaction of SV40 DNA synthesis requires a specific interaction between the SV40 origin and a virally encoded protein, the large T-antigen. After binding to the origin, T-antigen

cooperates with topoisomerase I and RP-A to unwind origin proximal DNA [47,48]. All subsequent steps in the replication process (initiation, elongation and termination) solely depend upon simian or human host proteins. Therefore, utilization of the SV40 *in vitro* DNA replication system has allowed researchers to determine the identities and functions of several proteins essential to mammalian DNA synthesis [49-51]. In particular, replication protein A (RP-A), DNA polymerase α-primase, DNA polymerase δ, proliferating cell nuclear antigen (PCNA), replication factor C (RF-C), maturation factor 1 (MF-1), DNA ligase I, and DNA topoisomerases I and II, actively participate in DNA synthesis *in vitro*. A brief description of each of these proteins participating in DNA synthesis *in vitro* follows.

RP-A

During SV40 DNA synthesis *in vitro*, RP-A, or human single-stranded DNA binding protein (SSB), stabilizes newly formed single-stranded regions created in template DNA by the helicase activity of the large T-antigen [48;52-54]. RP-A is purified from human cells as a tightly associated complex of three polypeptides with molecular masses of 70, 32 and 14 kDa [54]. Although the entire complex functions as an SSB, recent studies have localized the DNA binding activity of RP-A to the 70 kDa subunit [55]. RP-A probably participates in both the initiation and elongation stages of DNA synthesis. First, it has been shown that during the former stage of replication, RP-A is necessary for the presynthetic unwinding of SV40 origin containing DNA by the viral large T-antigen [56]. Second, a role for RP-A in the elongation stage is surmised from its ability to stimulate the activity of DNA polymerase δ in the presence of the accessory factors,

PCNA and RF-C [57]. Finally, there is a substantial body of evidence suggesting that RP-A may also act in DNA repair and recombination [58-60].

DNA Polymerase α-primase

DNA polymerase α-primase is purified from eukaryotic cells as a four polypeptide complex with subunits having molecular masses of 48, 58, 70 and 180 kDa [61]. DNA polymerase α activity resides in the 180 kDa subunit; whereas, DNA primase is composed of the 48 and 58 kDa subunits. The function of the 70 kDa polypeptide has not been fully determined; however, studies suggest that it may tether the polymerase α-primase complex to replication forks or serve as a connector between these two proteins [55;62]. During the pre-initiation unwinding reaction of the SV40 origin, DNA polymerase α-primase interacts with the viral large T-antigen and RP-A [63,64]. This physical interaction is required for the synthesis of a nascent RNA-DNA by polymerase α-primase on both the leading and lagging strand templates. According to one model for DNA replication, DNA primase continues to catalyze the synthesis of small oligoribonucleotide repeats on the lagging strand during the elongation stage, and these primers are subsequently extended by polymerase α to form Okazaki fragments [64].

DNA Polymerase δ

Another polymerase essential to DNA replication *in vitro* is DNA polymerase δ [65-67]. DNA polymerase δ is composed of two subunits: a 125 kDa polypeptide that possesses an intrinsic 3'-5' exonuclease activity and a 50 kDa polypeptide of unknown function [61]. Different from polymerase α, DNA polymerase δ is believed to conduct leading strand DNA replication during DNA chain elongation [49;64]. In support of this

proposed role, only short lagging strand SV40 DNA products are synthesized *in vitro* in replication reactions lacking purified polymerase δ [64]. The processivity of polymerase δ is stimulated approximately 100 fold by PCNA, which forms a sliding clamp around duplex DNA facilitating the continuous translocation of polymerase δ along the template [68]. Additionally, RF-C, in the presence of RP-A and PCNA, has been reported to cooperatively stimulate the activity of polymerase δ [57].

PCNA

The gene for PCNA encodes a single polypeptide of 28.7 kDa; however, PCNA migrates through denaturing polyacrylamide gels with an apparent molecular mass of 36 kDa [68]. Additionally, data from gel filtration and glycerol gradient sedimentation analyses demonstrate that native PCNA exists as a trimer, in which the three molecules adopt a ring-like or sliding clamp structure around duplex DNA [68]. The formation of this sliding clamp configuration by PCNA enables the protein to facilitate the continuous translocation of polymerase δ along template DNA during the elongation stage of DNA replication. Indeed, several studies have shown that in the absence of PCNA only early replicative lagging strand products are synthesized during SV40 DNA replication *in vitro* [66]. As polymerase δ depends on PCNA for its full activity and processivity, the latter protein has been designated an accessory factor for the polymerase [69,70]. Furthermore, that PCNA plays a vital role in cellular metabolism stems from the observation that many of the biochemical properties of the protein, i.e., molecular weight, isoelectric point and antigenicity, have been highly conserved during evolution [71].

RF-C

Another cellular factor essential to SV40 DNA replication *in vitro* is RF-C or activator 1. RF-C is isolated from human cells as a protein complex composed of five unique polypeptides with molecular masses of 140-145, 40, 38, 37 and 36 kDa [72-74]. The RF-C complex is an ATP-dependent, structure-specific DNA binding apparatus that recognizes the primer-template junction [75]. After binding to the 3' end of nascent DNA at the origin, RF-C displaces polymerase α-primase from the primer terminus and subsequently loads PCNA and polymerase δ onto DNA [49;64;76]. The assembly of the RF-C-PCNA-polymerase δ complex permits efficient and processive leading strand DNA synthesis for many thousands of nucleotides; while polymerase α-primase continues to prime and synthesize DNA discontinuously on the lagging strand template [49;57]. It has recently been proposed that RF-C facilitates coordinated synthesis of both the leading and lagging strands during DNA replication by acting as a connector or hinge between polymerases α and δ [57;64].

MF-1 and DNA Ligase I

Both MF-1 and DNA ligase I are essential for the maturation of daughter DNA molecules in SV40 DNA replication *in vitro* [45;64]. MF-1 is a 44 kDa structure-specific 5'-3' exo/endonuclease which is also known as flap-endonuclease 1 (FEN-1), RTH-1 and the human homologue of the *Schizosaccharomyces pombe* rad 2 gene product [77]. During Okazaki fragment processing, MF-1, along with RNase H1, remove initiator RNA primers from the lagging strand, allowing DNA polymerase to create a substrate for ligation [78]. DNA ligase I mediates this ligation reaction in which the adjacent 5' and 3'

ends of Okazaki fragments are covalently joined to produce form I closed circular daughter DNA molecules [64;79].

DNA Topoisomerases I and II

Human topoisomerase I is a monomeric 100 kDa nuclear protein that is essential to such cellular processes as DNA replication, transcription and recombination [80,81]. During the first stage of SV40 DNA synthesis *in vitro*, topoisomerase I cooperates with T-antigen and RP-A to extensively unwind the SV40 origin [48;53;82,83], and in later stages, the enzyme relaxes positive and negative DNA supercoils as they accumulate ahead of the replication fork [84]. Topoisomerase I alters DNA topology by introducing transient single-strand breaks into the DNA phosphodiester backbone, through which another DNA strand can pass, then rejoins these nicked strands in an energy-independent process [84;85]. During this breakage-reunion reaction, topoisomerase I forms a short-lived cleavable complex with DNA, in which the enzyme remains covalently linked to the 3' end of the nicked strand. Many topoisomerase I inhibitors, i.e., camptothecin (CPT), exert their cytotoxicity by stabilizing cleavable complex formation [85-87]. It is postulated that cell death results from irreparable DNA damage produced via the collision of moving replication and transcription forks with drug-stabilized topoisomerase I DNA cleavable complexes [85;88,89].

Topoisomerase II is a homodimeric nuclear enzyme that also relaxes supercoiled DNA and, in a unique reaction, facilitates the separation of intertwined DNA molecules. Specifically, topoisomerase II introduces transient double-strand breaks into the DNA phosphodiester backbone, allowing the passage of duplex DNA [84]. After strand passage, topoisomerase II catalyzes the religation of DNA double-strand breaks in an ATP

dependent reaction. Many anticancer agents that target topoisomerase II, i.e. etoposide (VP-16), inhibit this religation step and stabilize the formation of DNA topoisomerase II cleavable complexes [90;91]. Studies performed in intact CV-1 cells demonstrate that topoisomerase II plays an essential role as a swivelase in the late stages of SV40 chromosome replication, as VP-16 specifically inhibits the decatenation of daughter DNA molecules [88,92]. In addition to DNA replication, topoisomerase II has been reported to play critical roles in RNA transcription, chromosome condensation and segregation, and may tether cellular replication machinery to the nuclear matrix [93].

It has recently been found that two isoforms of topoisomerase II exist in mammalian cells, α (170 kDa) and β (180 kDa), which differ in their sensitivities to pharmacological agents and their levels of expression throughout the cell cycle [90;93]. Importantly, the α isoform of the enzyme is preferentially inhibited by cleavable complex forming topoisomerase II inhibitors (i.e., VP-16) [90].

DNA Polymerase ε and Poly(ADP-ribose) Polymerase

Although neither DNA polymerase ε nor poly(ADP-ribose) polymerase (PARP) is necessary for SV40 DNA replication *in vitro*, each has been shown to be essential for mammalian cell DNA synthesis. Therefore, a brief description of these polypeptides follows.

DNA Polymerase ε

Mammalian DNA polymerase ε is composed of a greater than 200 kDa catalytic subunit and a 55 kDa non-catalytic polypeptide; a smaller 140 kDa form of polymerase ε has also been purified from eukaryotic cells and may represent a proteolytic product of the

former [94]. DNA polymerase ε is not required for SV40 DNA synthesis *in vitro*; however, several reports demonstrate that the enzyme is essential to cellular DNA replication. For example, when the gene encoding the yeast homologue of DNA polymerase ε is mutated, the yeast cells fail to proliferate [95]. Also, it has been shown that DNA polymerase ε is photolabeled by newly made cellular DNA in CV-1 cells, indicating that the polymerase participates in the replication of nuclear chromosomes [96]. It is postulated that polymerase ε links the replication machinery with the S-phase checkpoint by acting as a sensor that coordinates transcriptional responses to DNA damage [97].

PARP

PARP is a 116 kDa enzyme that post-translationally modulates the structure and function of different nuclear proteins such as histones H1, H2B, H4, DNA topoisomerase I, DNA ligase II and DNA polymerase β by catalyzing their ADP-ribosylation [98]. This post-translational modification plays an auxiliary role in several nuclear processes such as DNA replication, DNA repair and cell differentiation [99]. A role for PARP in DNA synthesis is further supported by the observations that PARP activity is markedly enhanced in proliferating cells [100], newly replicated chromatin [101] and DNA fragments enriched in replication forks [102]. Although PARP is not necessary for DNA synthesis *in vitro*, the enzyme has been shown to sequester free 3' ends of Okazaki fragments during reconstituted SV40 DNA replication *in vitro*, thereby inhibiting their elongation by DNA polymerase α [103]. The binding of PARP to DNA termini suggests that the polymerase may act as a molecular nick sensor [99]. In this role, PARP would control the progression of the replication fork to allow the repair of damaged DNA prior to its duplication.

III. MULTIPROTEIN COMPLEXES FOR DNA REPLICATION

Although the identities and functions of many proteins required for DNA synthesis have been determined, their functional organization permitting the efficient replication of DNA has not been determined. Mammalian cell DNA replication represents an intricate and complex process. First, the mammalian chromosome is a complex nucleoprotein structure composed of DNA containing multiple origins of replication as well as proteins that must be duplicated along with DNA to maintain chromosome organization [104]. Second, chromosomal replication occurs in a precise, temporally and spatially regulated manner within individual replication units or replicons [105]. As mammalian cells contain multiple chromosomes, each possessing many replication origins, chromosomal replication must be a highly coordinated process; one that does not merely occur by random collisions between soluble enzyme factors and substrates.

It has recently been postulated that the DNA synthetic enzymes and non-enzymic proteins, whose concerted action is required for efficient DNA replication, may be organized into a multiprotein complex. In support of this hypothesis, several reports have described the isolation of large macromolecular complexes of replication-essential polypeptides from the extracts of eukaryotic cells [45;104;106-108]. For example, a two million dalton complex that possesses DNA polymerase, DNA primase, DNA ligase and topoisomerase II activities and functions to replicate the extrachromosomal, 2 micron yeast plasmid DNA *in vitro* has been isolated from yeast cells [109]. Additionally, a megacomplex of enzymes involved in dNTP synthesis and DNA replication, known as replitase, has been purified from Chinese hamster embryo fibroblast cells (CHEF/18)

[110]. Replitase produces deoxynucleoside triphosphates, channels them to the microvicinity of DNA replication, and effects their polymerization. Furthermore, a protein fraction predominantly enriched for DNA polymerases α and δ, RP-A, DNA topoisomerase II and ors (origin enriched sequence) binding activity, which binds to the *ors* 8 mammalian autonomously replicating DNA sequence, has been purified from human cervical cancer (HeLa) cells [111]. This putative multiprotein complex is capable of supporting the replication of *ors* 8 plasmid DNA *in vitro*. Finally, a multiprotein complex (RC complex) composed of DNA polymerase α-primase, DNA polymerase δ and RF-C has recently been purified from calf thymus [112]. The RC complex is capable of polymerizing primed and unprimed single-stranded M13 DNA templates *in vitro*.

Our laboratory was the first to isolate and characterize a multiprotein DNA replication complex, designated the DNA synthesome, from human cervical cancer (HeLa) and murine mammary carcinoma (FM3A) cells that fully supports origin specific and large T-antigen dependent papovavirus DNA replication *in vitro* [45;104;113-115]. As described in later chapters of this thesis, we have also successfully isolated the DNA synthesome from human breast cancer (MDA MB-468) cells as well as from biopsied human breast tumor tissue and xenografts from nude mice injected with the breast cancer cell line MCF-7 [116]. The DNA products synthesized by the DNA synthesome consist of monomeric circular form I and II DNAs as well as topological and replicative intermediates. Furthermore, the majority (80-90%) of these products are resistant to digestion by *DpnI*, which is consistent with the criteria for semi-conservative replication of full-length DNA. In addition to the viral large T-antigen and SV40 origin sequences, the replication activity of the DNA synthesome is dependent on Mg^{2+}, ribonucleoside

triphosphates, deoxyribonucleotide triphosphates and a renewable source of ATP, provided by phosphocreatine kinase and phosphocreatine [45]. These requirements for DNA synthesome mediated *in vitro* DNA replication are comparable to those observed for cells permissive for SV40 infection [117] as well as for crude extracts prepared from cells that are permissive for SV40 DNA synthesis [118]. These results suggest that the DNA synthesome represents the replication machinery of intact cells.

We have reported that the DNA synthesome retains its ability to replicate papovavirus DNA after its additional purification by anion-exchange chromatography and sucrose or glycerol gradient sedimentation [113,114;116]. In addition, the integrity of the multiprotein complex is maintained after treatment with salt, detergents, RNase, DNase and electrophoresis through native polyacrylamide gels [113;115]. These results suggest that the association of the protein components with one another is independent of non-specific interactions with other cellular macromolecules. The proteins and enzymes that copurify with the DNA synthesome include: DNA polymerase α-primase, DNA polymerases δ and ε, PCNA, RF-C, RP-A, DNA helicases I and IV, DNA ligase I, PARP and DNA topoisomerases I and II.

IV. THE DNA SYNTHESOME: A MODEL FOR STUDYING THE MECHANISMS OF ACTION OF ANTICANCER AGENTS

As the DNA synthesome is a replication-competent multiprotein complex, it may serve as a novel model for examining the mechanisms of action of anticancer agents that target the DNA synthetic process. In support of this hypothesis, we have previously reported that the replication activity of the DNA synthesome purified from HeLa cells is as

sensitive to camptothecin poisoning as is intact HeLa cell DNA synthesis [119]. Specifically, we found that DNA synthesome-associated topoisomerase I activity as well as *in vitro* and intact HeLa cell DNA replication activities are each inhibited 50% by 0.05 μM camptothecin, and increasing concentrations of the agent causes an accumulation of DNA synthesome-associated topoisomerase I cleavable complexes [119]. These results are consistent with the premise that camptothecin inhibits deoxyribonucleic acid synthesis by selectively trapping topoisomerase I in a ternary cleavable complex. In another study from our laboratory, we reported that the HeLa cell DNA synthesome can utilize 1-β-D-arabinofuranosyl cytosine (ara-C) as a substrate for *in vitro* DNA synthetic reactions [120]. During synthesome-mediated DNA replication, ara-C is incorporated into internucleotide positions in nascent and full length SV40 daughter DNA molecules [120]; a result consistent with findings obtained by others studying the effects of ara-C on intact cell DNA synthesis [121-124].

Several advantages are conferred by employing the DNA synthesome as a model for examining the mechanisms of action of anticancer agents, as compared to the use of conventional systems. For example, employment of the DNA synthesome as a drug model permits the study of the interaction of an anticancer agent with the entire DNA replication apparatus and not just individual proteins involved in DNA synthesis. Indeed, studies performed with individual DNA replication proteins purified from human or animal cells possess numerous limitations: 1) not all of the proteins and enzymes involved in mammalian cell DNA replication have been identified, so the target-protein activity of a given anticancer agent could be missing from the current panel of known DNA replication proteins [125]; 2) the interaction of an anticancer agent with its protein target may be

different when the protein is associated with other cellular components as opposed to when it acts alone in the presence of drug [125]. Additionally, the use of the DNA synthesome as a drug model allows the examination of the mechanisms of action of anticancer agents in the absence of any enzymatic processes that may secondarily affect DNA synthesis. This is a problem inherent to drug studies performed in systems using crude cell extracts.

Irinotecan (CPT-11; active metabolite, SN-38) (Figure 2) and etoposide (VP-16) (Figure 3) are two DNA topoisomerase inhibitors that have demonstrated efficacy in the treatment of several types of cancer, including breast cancer [126-128]. SN-38 possesses a unique mechanism of action in that it traps nuclear topoisomerase I in a ternary (drug-enzyme-DNA) cleavable complex [91]. In this state, topoisomerase I cannot perform its DNA single strand nicking-resealing function required for the relaxation of supercoiled DNA. Similarly, VP-16 traps topoisomerase II in a cleavable complex which prevents the enzyme from introducing transient double-strand breaks into the DNA phosphodiester backbone, an action required for the segregation of newly formed daughter DNA molecules [85;91]. For both SN-38 and VP-16, cleavable complex formation represents an early step in a cascade of events that eventually leads to cell death [85;91]. Currently, the precise molecular mechanisms by which drug-stabilized cleavable complex formation leads to irreparable DNA damage are unresolved. We hypothesize that the use of the breast cell DNA synthesome as a model for examining the mechanisms of action of SN-38 and VP-16 will help elucidate their mechanisms of action and aid the development of improved analogues of these agents for breast cancer therapy.

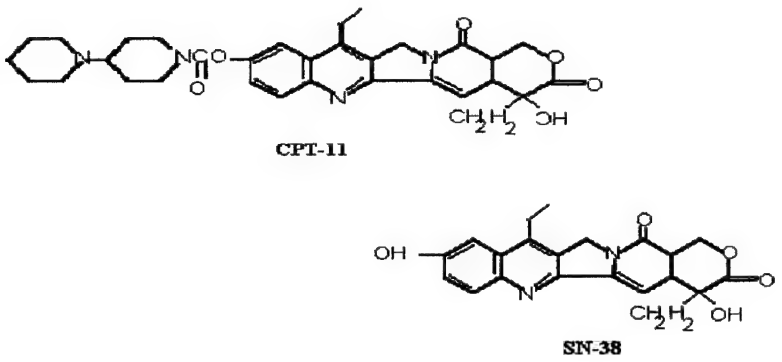


Figure 2. Chemical structure of irinotecan (CPT-11/SN-38).

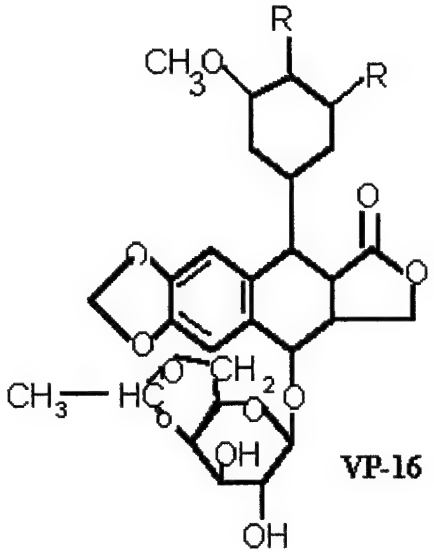


Figure 3. Chemical structure of etoposide (VP-16).

V. OBJECTIVES

In conclusion, the aggressive progression of breast cancer is characterized by a high proliferation phenotype and the accumulation of an extensive level of DNA damage within tumor cells [8;12;29,30]. As tumor cell kinetics can independently predict metastatic potential, relapse-free and overall patient survival [33;36], understanding the molecular mechanisms of breast cell DNA replication is critical to the development of improved anti-breast cancer therapies. Toward this end of better understanding the process of human breast cell DNA replication, the specific aims of this thesis project included: **(1)** to purify and characterize the DNA synthesome from human breast cancer (MDA MB-468) cells as well as from biopsied human breast tumor tissue and xenografts from nude mice injected with the breast cancer cell line MCF-7; and **(2)** to determine the protein-protein interactions between several of the core components of the breast cell DNA synthesome. The final aim of this thesis project was **(3)** to demonstrate that the breast cell DNA synthesome represents a novel model for examining the mechanisms of action of SN-38 and VP-16.

References

1. Landis, S.H., Murray, T., Bolden, S. and Wingo, P.A. (1998) Cancer statistics, 1998. CA: Cancer J. for Clin. **48**: 6-29.

2. Davis, A.R. Breast cancer: the search for new prognostic markers. Br. J. Biomed. Sci. **53**: 157-161; 1996.

3. Ozbun, M.A. and Butel, J.S. Tumor suppressor p53 mutations and breast cancer: a critical analysis. Adv. Cancer Res. **66**: 71-141; 1995.

4. Wellings, S.R. Development of human breast cancer. Adv. Cancer Res. **31**: 287-314; 1980.

5. DuPont, W.D. and Page, D.L. Risk factors for breast cancer in women with proliferative breast disease. New Engl. J. Med. **312**: 146-151; 1985.

6. Silverberg, S.G. and Chitale, A.R. Assessment of significance of proportion of intraductal and infiltrating tumor growth in ductal carcinoma of the breast. Cancer **32**: 830-837; 1973.

7. O'Connell, P.; Pekkel, V.; Fuqua, S.; Osborne, C.K.; Allred, D.C. Molecular genetic studies of early breast cancer evolution. Br. Cancer Res. Trtmnt. **32**: 5-12; 1994.

8. Dairkee, S.H.; Smith. S.H. Genetic analysis of breast cancer progression. J. Mamm. Gl. Biol. and Neoplasia **1**: 139-151; 1996.

9. Sager, R. Tumor suppressor genes: the puzzle and the promise. Science: **246**: 1406-1412; 1989.

10. Kreipe, H.; Feist, H.; Fischer, L.; Felgner, J.; Heidorn, K.; Mettler, L.; Parwaresch, R. Amplification of c-*myc* but not of c-*erb*B-2 is associated with high proliferative capacity in breast cancer. Cancer Res. **53**: 1956-1961; 1993.

11. Pietilainen, T.; Lipponen, P.; Aaltomaa, S.; Eskelinen, M.; Kosma, V.M.; Syrjanen, K. Expression of retinoblastoma gene protein (Rb) in breast cancer as related to established prognostic factors and survival. Eur. J. Cancer **31A**: 329-333; 1995.

12. Chen, L.C.; Neubauer, A.; Kurisu, W.; Waldman, F.M.; Ljung, B.M.; Goodson, W.; Goldman, E.S.; Moore, D.; Balazs, M.; Liu, E. LOH on the short arm of chromosome 17 is associated with high proliferative capacity and DNA aneuploidy in primary human breast cancer. Proc. Natl. Acad. Sci. USA **88**: 3847-3851; 1991.

13. Koreth, J.; Bethwaite, P.B.; McGee, J.O. Mutations at chromosome 11q23 in human non-familial breast cancer: a microdissection, microsatellite analysis. J. Pathol. **176**: 11-18; 1995.

14. Liu, E.; Dollbaum, C.; Scott, G.; Rochlitz, C.; Benz, C.; Smith, H.S. Molecular lesions involved in the progression of human breast cancer. Oncogene **3**: 323-327; 1988.

15. Zhuang, Z.; Merino, M.J.; Chuaqui, R.; Liotta, L.A.; Emmert-Buck, M.R. Identical allelic loss on chromosome 11q13 in microdissected in situ and invasive human breast cancer. Cancer Res. **55**: 467-471; 1995.

16. Aldaz, C.M.; Chen, T.; Sahin, A.; Cunningham, J.; Bondy, M. Comparative allelotypes of in situ and invasive human breast cancer: high frequency of microsatellite instability in lobular breast carcinomas. Cancer Res. **55**: 3976-3981; 1995.

17. Osborne, R.J.; Merlo, G.R.; Mitsudomi, T.; Venesio, T.; Liscia, D.S.; Cappa, A.P.; Chiba, I.; Takahashi, T.; Nau, M.M.; Callahan, R. Mutations in the p53 gene in primary human breast cancer. Cancer Res. **51**: 6194-6198; 1991.

18. Levine, A.J. p53, the cellular gatekeeper for cell growth and division. Cell **88**: 323-331; 1997.

19. Selivanova, G. and Wiman, K.G. p53: a cell cycle regulator activated by DNA damage. Adv. Cancer Res. **66**: 143-180; 1995.

20. Lowe, S.W.; Ruley, H.E.; Jacks, T; Housman, D.E. p53 dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell **74**: 957-967; 1993.

21. Clark, A.R.; Purdie, C.A.; Harrison, D.J.; Morris, R.G.; Bird, C.C.; Hooper, M.L.; Wyllie, A.H. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature **362**: 849-852; 1993.

22. Deng, G.; Chen, L.C.; Schott, D.R.; Thor, A.; Bhargava, V.; Ljung, B.M.; Chew, K.; Smith, H.S. LOH and p53 gene mutations in breast cancer. Cancer Res. **54**: 499-505; 1994.

23. Eyfjord, J.E.; Thorlacius, S.; Steinarsdottir, M.; Valgardsdottir, R.; Ogmundsdottir, H.M.; Anamthawat-Jonsson, K. p53 abnormalities and genomic instability in primary human breast carcinomas. Cancer Res. **55**: 646-651; 1995.

24. Pellegata, N.S.; Ranzani, G.N. The significance of p53 mutations in human cancers. Eur. J. Histochem. **40**: 273-282; 1996.

25. Ozer, E.; Canda, T.; Kuyucuodlu, F. p53 mutations in bilateral breast carcinomas. Correlation with Ki-67 expression and the mean nuclear volume. Cancer Lett. **122**: 101-106; 1998.

26. Harris, A.L. p53 expression in human breast cancer. Adv. Cancer Res. **59**: 69-88; 1992.

27. Thor, A.D.; Moore, D.H.; Edgerton, S.M.; Kawasaki, E.S.; Reihsaus, E.; Lynch, H.T.; Marcus, J.N.; Schwartz, L.; Chen, L.C.; Mayall, B.H. Accumulation of p53 tumor

suppressor gene protein: an independent marker of prognosis in breast cancers. J. Natl. Cancer Inst. **84**: 845-855; 1992.

28. Horak, E.; Smith, K.; Bromley, L.; LeJeune, S.; Greenall, M.; Lane, D.; Harris, A.L. Mutant p53, EGF receptor and cerbB2 expression in human breast cancer. Oncogene **6**: 2277-2284; 1991.

29. D'Agnano, I.; Bucci, B.; Mottolese, M.; Benevolo, M.; Conti, L.; Botti, C.; Vecchione, A.; Casillo, S.; Zupi, G. DNA ploidy, cell kinetics and EGFR and HER2/neu oncoprotein expression in primary operable breast cancer. Ann. New York Acad. Sci. **784**: 472-481; 1993.

30. Borg, A.; Baldetorp, B.; Ferno, M.; Killander, D.; Olsson, H.; Sigurdsson, H. ErbB2 amplification in breast cancer is associated with a high rate of proliferation. Oncogene **6**: 137-143; 1991.

31. O'Reilly, S.M.; Barnes. D.M.; Camplejohn, R.S.; Bartkova, J.; Gregory, W.M.; Richards, M.A. The relationship between cerbB2 expression, S phase fraction and prognosis in breast cancer. Br. J. Cancer **63**: 444-446; 1991.

32. Sutherland, R.L.; Hamilton, J.A.; Sweeney, K.J.E.; Watts, C.K.W.; Musgrove, E.A. Expression and regulation of cyclin genes in breast cancer. Acta Oncol. **34**: 651-656; 1995.

33. Clark, G.M.; Dressler, L.G.; Owens, M.A.; Pounds, G.; Oldaker, T.; McGuire, W.L. Prediction of relapse or survival in patients with node-negative breast cancer by DNA flow cytometry. New Engl. J. Med. **320**: 627-633; 1989.

34. Gasparini, G.; Boracchi, P.; Verderio, P.; Bevilacqua, P. Cell kinetics in human breast cancer: comparison between the prognostic value of the cytofluorometric S-phase fraction

and that of the antibodies to Ki-67 and PCNA antigens detected by immunocytochemistry. Int. J. Cancer **57**: 822-829; 1994.

35. Silvestrini, R. Feasibility and reproducibility of the ^{3}H-thymidine labeling index in breast cancer. Cell Prolif. **24**: 437-445; 1991.

36. Witzig, T.E.; Ingle, J.N.; Cha, S.S.; Schaid, D.J.; Tabery, R.L.; Wold, L.E.; Grant, C.; Gonchoroff, N.J.; Katzmann, J.A. DNA ploidy and the percentage of cells in S-phase as prognostic factors for women with lymph node negative breast cancer. Cancer **74**: 1752-1761; 1994.

37. Remvikos, Y.; Mosseri, V.; Zajdela, A.; Fourquet, A.; Durand, J.C.; Pouillart, P.; Magdelenat, H. Prognostic value of the S-phase fraction of breast cancers treated by primary radiotherapy or neoadjuvant chemotherapy. Annals New York Acad. Sci. **698**: 193-203; 1993.

38. Tubiana, M.; Pejovic, M.H.; Koscielny, S.; Chavaudra, N.; Malaise, E. Growth rate, kinetics of tumor cell proliferation and long-term outcome in human breast cancer. Int. J. Cancer **44**: 17-22; 1989.

39. Clark, G.M.; McGuire, W.L. Prognosis of breast cancer patients: how to use what? Recent Res. Cancer Rev. **127**: 55-60; 1993.

40. McGuire, W.L.; Clark, G.M. Prognostic factors and treatment decisions in axillary node negative breast cancer. New Engl. J. Med. **326**: 1756-1761; 1992.

41. Coll, J.M.; Sekowski, J.W.; Hickey, R.J.; Schnaper, L.; Yue, W.; Brodie, A.M.H.; Uitto, L.; Syvaoja, J.E.; Malkas, L.H. The human breast cell DNA synthesome: Its purification from tumor tissue and cell culture. Oncol. Res. **8**: 435-447; 1996.

42. Popanda, O.; Fox, G,; Thielmann, H.W. DNA polymerases α, δ and ε of Novikoff hepatoma cells differ from those of normal rat liver in physicochemical and catalytic properties. J. Mol. Med. **73**: 259-268; 1995.

43. Li, J.J. and Kelly, T.J. Simian virus 40 DNA replication *in vitro*. Proc. Natl. Acad. Sci. USA **81**: 6973-6977; 1984.

44. Li, J.J. and Kelly, T.J. Simian virus 40 DNA replication in vitro: specificity of initiation and evidence for bidirectional replication. Mol. Cell. Biol. **5**: 1238-1246; 1985.

45. Hickey, R.J. and Malkas, L.H. (1997) Mammalian cell DNA replication. In: *Critical Reviews in Eukaryotic Gene Expression*, **7**: 125-157.

46. DePamphilis, M.L. and Bradley, M.K. (1986) Replication of SV40 and Polyoma Virus Chromosomes. Salzman, N.P. (ed.) In: *The Papovaviridae Volume I: The Polyomavirus*, pp. 99-246, Plenum Press, New York, NY.

47. Boroweic, J.A.; Dean, F.B.; Bullock, P.A.; Hurwitz, J. Binding and unwinding: How T-antigen engages the SV40 origin of DNA replication. Cell **60**:181-184; 1990.

48. Wold, M.S.; Li, J.J.; Kelly, T.J. Initiation of simian virus 40 DNA replication *in vitro*: large tumor antigen and origin-dependent unwinding of the template. Proc. Natl. Acad. Sci. USA **84**: 3643-3647; 1987.

49. Tsurimoto, T.; Stillman, B. Replication factors required for SV40 DNA replication *in vitro*. J. Biol. Chem. **266**: 1961-1968; 1991.

50. Hurwitz, J.; Dean, F.B.; Kwong, A.D.; Lee, S-H. The *in vitro* replication of DNA containing the SV40 origin. J. Biol. Chem. **30**: 18043-18046; 1990.

51. Wobbe, C.R.; Dean, F.B.; Weissbach, L.; Hurwitz, J. *In vitro* replication of duplex circular DNA containing the simian virus 40 DNA origin site. Proc. Natl. Acad. Sci. USA **82**: 5710-5714; 1985.

52. Wobbe, C.R.; Weissbach, L.; Borowiec, J.A.; Dean, F.B.; Murakami, Y.; Bullock, P.; Hurwitz, J. Replication of simian virus 40 origin-containing DNA *in vitro* with purified proteins. Proc. Natl. Acad. Sci. USA **84**: 1834-1838; 1987.

53. Dodson, M.; Dean, F.B.; Bullock, P.; Echols, H.; Hurwitz, J. Unwinding of duplex DNA from the SV40 origin of replication by T-antigen. Science **238**: 964-967; 1987.

54. Kenny, M.K.; Schlegel, U.; Furneaux, H.; Hurwitz, J. The role of human single-stranded DNA binding protein and its individual subunits in simian virus 40 DNA replication. J. Biol. Chem. **265**: 7963-7967.

55. Wold, M.S.; Weinberg, D.H.; Virshup, D.M.; Li, J.J.; Kelly, T.J. Identification of cellular proteins required for simian virus 40 DNA replication. J. Biol. Chem. **264**: 2801-2809; 1989.

56. Wold, M.S. and Kelly, T.J. Purification and characterization of replication protein A, a cellular protein required for *in vitro* replication of simian virus DNA. Proc. Natl. Acad. Sci. USA **85**: 2523-2527; 1988.

57. Tsurimoto, T. and Stillman, B. Multiple replication factors augment DNA synthesis by the two eukaryotic DNA polymerases, alpha and delta. EMBO J **8**: 3883-3889; 1989.

58. Coverley, D.; Kenny, M.K.; Lane, D.P.; Wood, R.D. A role for the human single-stranded DNA binding protein HSSB/RP-A in an early stage of nucleotide excision repair. Nucl. Acids Res. **20**: 3873-3880; 1992.

59. Moore, S.P.; Erdile, L.; Kelly, T.; Fishel, R. The human homologous pairing protein HPP-1 is specifically stimulated by the cognate single-stranded binding protein hRP-A. Proc. Natl. Acad. Sci. USA **88**: 9067-9071;1991.

60. Longhese, M.P.; Plevani, P.; Lucchini, G. Replication factor A is required *in vivo* for DNA replication, repair and recombination. Mol. Cell. Biol. **14**: 7884-7890; 1994.

61. Wang, T.S. Eukaryotic DNA polymerases. Annual Rev. Biochem. **60**: 513-552; 1991.

62. Plevani, P.; Foiani, M.; Valsasnini, P.; Badaracco, G.; Cheriathundam, E.; Chang, L.M. Polypeptide structure of DNA primase from a yeast DNA polymerase-primase complex. J. Biol. Chem. **260**: 7102-7107; 1985.

63. Murakami, Y.; Hurwitz, J. DNA polymerase alpha stimulates the ATP-dependent binding of simian virus tumor T antigen to the SV40 origin of replication. J. Biol. Chem. **268**: 11018-11027; 1993.

64. Waga, S.; Stillman, B. Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication *in vitro*. Nature **369**: 201-212; 1994.

65. Melendy, T.; Stillman, B. Purification of DNA polymerase delta as an essential simian virus 40 DNA replication factor. J. Biol. Chem. **266**: 1942-1949; 1991.

66. Prelich, G.; Stillman, B. Coordinated leading and lagging strand synthesis during SV40 DNA replication *in vitro* requires PCNA. Cell **53**: 117-126; 1988.

67. Prelich, G.; Tan, C-K; Mikostura, M.; Mathews, B.; So, A.G.; Downey, K.M.; Stillman, B. Functional identity of proliferating cell nuclear antigen and a DNA polymerase delta auxiliary protein. Nature (London) **326**: 517-520; 1987.

68. Brand, S.R.; Bernstein, R.M.; Mathews, M.B. Trimeric structure of human proliferating cell nuclear antigen: implication for enzymatic function and autoantibody recognition. J. Immunol., **15**: 3070-3079; 1994.

69. Bravo, R.; Frank, R.; Lundell, P.A.; MacDonald-Bravo, H. Cyclin/PCNA is the auxiliary protein of DNA polymerase delta. Nature (London) **326**: 515-517; 1987.

70. Tan, C-K; Castillo, C.; So, A-G; Downey, K.M. An auxiliary protein for DNA polymerase delta from fetal calf thymus. J. Biol. Chem. **261**: 12310-12316; 1986.

71. Morris, G.F. and Mathews, M.B. Regulation of proliferating cell nuclear antigen during the cell cycle. J. Biol. Chem. **23**: 13856-13864; 1989.

72. Bunz, F.; Kobayashi, R.; Stillman, B. cDNAs encoding the large subunit of human replication factor C. Proc. Natl. Acad. Sci. USA **90**: 11014-11018; 1993.

73. Pan, Z-Q; Chen, M.; Hurwitz, J. The subunits of activator-1 (replication factor C) carry out multiple functions essential for proliferating cell nuclear antigen-dependent DNA synthesis. Proc. Natl. Acad. Sci. USA **90**: 6-10; 1993.

74. Chen, M.; Pan, Z-Q; Hurwitz, J. Studies of the cloned 37 kDa subunit of activator 1 (replication factor C) of HeLa cells. Proc. Natl. Acad. Sci. USA **89**: 5211-5215; 1992.

75. Fotedar, R.; Mossi, R.; Fitzgerald, P.; Rouselle, T.; Maga, G.; Brickner, H.; Messier, H.; Kasibhatla, S.; Hubscher, U.; Fotedar, A. A conserved domain of the large subunit of replication factor C binds PCNA and acts like a dominant negative inhibitor of DNA replication in mammalian cells. EMBO J **15**: 4423-4433.

76. Podust, L.M.; Podust, V.N.; Sogo, J.M.; Hubscher, U. Mammalian DNA polymerase auxiliary proteins: analysis of replication factor C-catalyzed proliferating cell nuclear antigen loading onto circular double stranded DNA. Mol. Cell. Biol. **15**: 3072-3081; 1995.

77. Huang, L.; Rumbaugh, J.A.; Murante, R.S.; Lin, R.J.R.; Rust, L.; Bambara, R.A. Role of calf RTH-1 nuclease in removal of 5'-ribonucleotides during okazaki fragment processing. Biochem. **35**: 9266-9277; 1996.

78. Turchi, J.J.; Huang, L.; Murante, R.S.; Kim, Y.; Bambara, R.A. Enzymatic completion of mammalian lagging strand DNA replication. Proc. Natl. Acad. Sci. USA **91**: 9803-9807; 1994.

79. Waga, S.; Bauer, G.; Stillman, B. Reconstitution of complete SV40 DNA replication with purified replication factors. J. Biol. Chem. **269**: 10923-10934; 1994.

80. Pommier, Y. Diversity of DNA topoisomerases I and inhibitors. Biochimie **80**: 255-270; 1998.

81. Liu, L.F. and Miller, K.G. Eukaryotic DNA topoisomerases: two forms of type I DNA topoisomerases from HeLa cell nuclei. Proc. Natl. Acad. Sci. USA **78**: 3487-3491; 1981.

82. Borowiec, J.A.; Hurwitz, J. Localized melting and structural changes in the SV40 origin of replication induced by T-antigen. EMBO J. **7**: 3149-3158; 1988.

83. Dean, F.B.; Bullock, P.; Murakami, Y.; Wobbe, C.R.; Weissbach, L.; Hurwitz, J. Simian virus 40 (SV40) DNA replication: SV40 large T-antigen unwinds DNA containing the SV40 origin of replication. Proc. Natl. Acad. Sci. USA **84**: 16-20; 1987.

84. Wang, J.C. DNA topoisomerases. Annu. Rev. Biochem. **54**: 665-697; 1985.

85. Liu, L.F. DNA topoisomerase poisons as antitumor drugs. Annu. Rev. Biochem. **58**: 351-375; 1989.

86. Hsiang, Y.H. and Liu, L.F. Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. Cancer Res. **48**: 1722-1726; 1988.

87. Hsiang, Y.H., Hertzberg, R., Hecht, S. and Liu, L.F. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J. Biol. Chem. **260**: 14873-14878; 1985.

88. Snapka, R.M. Topoisomerase inhibitors can selectively interfere with different stages of simian virus 40 DNA replication. Mol. Cell. Biol. **6**: 4221-4227; 1986.

89. Ryan, A.J.; Squires, S.; Strutt, H.L.; Evans, A.; Johnson, R.T. Different fates of camptothecin-induced replication fork associated double-strand DNA breaks in mammalian cells. Carcinogen. **15**: 823-828; 1994.

90. Qiu, J.; Catapano, C.V.; Fernandes, D.J. Formation of topoisomerase II alpha complexes with nascent DNA is related to VM-26 induced cytotoxicity. Biochem. **35**: 16354-16360; 1996.

91. Schneider, E.; Hsiang, Y.H.; Liu, L.F. DNA topoisomerases as anticancer drug targets. Adv. Pharmacol. **21**: 149-183; 1990.

92. Snapka, R.M.; Powelson, M.A.; Strayer, A.M. Swiveling and decatenation of replicating simian virus 40 genomes *in vivo*. Molec. Cell Biol. **8**: 515-521; 1988.

93. Anderson, H.J.; Roberge, M. DNA topoisomerase II: a review of its involvement in chromosomal structure, DNA replication, transcription and mitosis. Cell Biol. Int. Rep. **16**: 717-724; 1992.

94. Uitto, L.; Halleen, J.; Hentunen, T.; Hoyhtya, M.; Syvaoja, J.E. Structural relationship between DNA polymerases epsilon and epsilon* and their occurrence in eukaryotic cells. Nucl. Acid Res. **23**: 244-247; 1995.

95. Morrison, A.; Araki, H.; Clark, A.B.; Hamatake, R.B.; Sugino, A. A third essential DNA polymerase in S. *cerevisiae*. Cell **62**: 1143-1151; 1990.

96. Zlotkin, T.; Kaufmann, G.; Jiang, Y.; Lee, M.Y.W.T; Uitto, L.; Syvaoja, J.; Dornreiter, I.; Fanning, E.; Nethanel, T. DNA polymerase epsilon may be dispensable for SV40 but not cellular DNA replication. EMBO J. **9**: 2298-2305; 1996.

97. Navas, T.A.; Zhou, Z.; Elledge, S.J. DNA polymerase ε links the DNA replication machinery to the S phase checkpoint. Cell **80**:29-39; 1995.

98. Simbulan, C.M.G.; Suzuki, M.; Izuta, S.; Sakurai, T.; Savoysky, E.; Kojima, K.; Miyahara, K.; Shizuta, Y.; Yoshida, S. Poly(ADP-ribose) polymerase stimulates DNA polymerase alpha by physical association. J. Biol. Chem. **268**: 93-99; 1993.

99. Simbulan-Rosenthal, C.M.; Rosenthal, D.S.; Hilz, H.; Hickey, R.J.; Malkas, L.H.; Applegren, N.; Wu, Y.; Bers, G.; Smulson, M.E. The expression of poly(ADP-ribose) polymerase during differentiation-linked DNA replication reveals that it is a component of the multiprotein DNA replication complex. Biochem. **35**: 11622-11633; 1996.

100. Lehman, A.R.; Kirk-Bell, S.; Shall, S.; Whish, W.J. Regulation of DNA synthesis in macrophages infected with simian virus 40. Exp. Cell Res. **83**: 63-72; 1974.

101. Anachkova, B.; Russev, G.; Poirier, G.G. DNA replication and poly(ADP-ribose) polymerization of chromatin. Cytobios **59**: 19-28; 1989.

102. deMurcia, G.; Jongstra-Bilen, J.; Ittel, M.E.; Mandel, P; Delain, E. Poly(ADP-ribose) polymerase automodification and interaction with DNA: electron microscopic visualization. EMBO J. **2**: 543-548; 1983.

103. Eki, T. and Hurwitz, J. Influence of poly(ADP-ribose) polymerase on the enzymatic synthesis of SV40 DNA. J. Biol. Chem. **15**: 3087-3100; 1991.

104. Malkas, L.H.; Hickey, R.J.; Baril, E.F. Multienzyme complexes for DNA synthesis in eukaryotes: P-4 revisited. In: Strauss, P.R. and Wilson, S.H. (eds.) "The Eukaryotic

Nucleus: Molecular Biochemistry and Macromolecular Assemblies." New Jersey, The Telford Press; 1990a: pp. 45-68.

105. DePamphilis, M.L.; Wasserman, P.M. Replication of eukaryotic chromosomes: a close-up of the replication fork. Annu. Rev. Biochem. **49**: 627-666; 1982.

106. Mathews, C.K. and Slabaugh, M.B. Eukaryotic DNA metabolism: Are deoxyribonucleotides channeled to replication sites? Exp. Cell Res. **162**: 285-295; 1986.

107. Fry, M.; Loeb, L.A. "Animal Cell DNA Polymerases." Boca Raton, FL: CRC Press; 1986.

108. Malkas, L.H.; Hickey, R.J.; Li, C.J.; Pedersen, N.; Baril, E.F. A 21S enzyme complex from HeLa cells that functions in simian virus 40 DNA replication *in vitro*. Biochem. **29**: 6362-6374; 1990b.

109. Jazwinski, S.M. and Edelman, G.M. Evidence for participation of a multiprotein complex in yeast DNA replication *in vitro*. J. Biol. Chem. **259**: 6852-6857; 1984.

110. Reddy, G. and Fager, R. Replitase: a complex integrating dNTP synthesis and DNA replication. Crit. Rev. Euk. Gene Exp. **3**: 255-277; 1993.

111. Ruiz, M.T.; Pearson, C.E.; Nielsen, T.; Price, G.B.; Zannis-Hadjopoulos, M. Cofractionation of HeLa cell replication proteins with *ors*-binding activity. J. Cell. Biochem. **58**: 221-236; 1995.

112. Maga, G. and Hubscher, U. DNA replication machinery: functional characterization of a complex containing DNA polymerase alpha, DNA polymerase delta and replication factor C suggests an asymmetric DNA polymerase dimer. Biochem. **35**: 5764-5777; 1996.

113. Wu, Y.; Hickey, R.J.; Lawlor, K.; Wills, P.; Yu, F.; Ozer, H.; Starr, R.; Quan, J-Y; Lee M.Y.W.T.; Malkas, L.H. A 17S multiprotein form of murine cell DNA polymerase mediates polyomavirus DNA replication *in vitro*. J. Cell. Biochem. **54**: 32-46; 1994.

114. Applegren, N.; Hickey, R.J.; Kleinschmidt, A.M.; Zhou, Q.; Coll, J.M.; Wills, P.; Swaby, R.; Wei, Y.; Quan, J.Y.; Lee, M.Y.W.T.; Malkas, L.H. Further characterization of the human cell multiprotein DNA replication complex. J. Cell. Biochem. **59**: 91-107; 1995.

115. Tom, T.; Hickey, R.J.; Malkas, L.H. Identification of multiprotein complexes containing DNA replication factors by native immunoblotting of HeLa cell protein preparations with T-antigen-dependent SV40 DNA replication activity. J. Cell. Biochem. **63**: 259-267; 1996.

116. Coll, J.M.; Sekowski, J.W.; Hickey, R.J.; Schnaper, L.; Yue, W.; Brodie, A.M.H.; Uitto. L.; Syvaoja, J.E.; Malkas, L.H. The human breast cell DNA synthesome: Its purification from tumor tissue and cell culture. Oncol. Res. **8**: 435-447; 1996.

117. DePamphilis, M.L.; Bradley, M.K. Replication of SV40 and polyoma virus chromosomes. In: Salzman, N.P. (ed.): "The Papovaviridae Volume I: The Polyomavirus" pp. 99-246, Plenum Press, New York, NY; 1986.

118. Decker, R.S.; Yamaguchi, M.; Possenti, R.; Bradley, M.; DePamphilis, M.L. *In vitro* initiation of DNA replication in SV40 chromosomes. J. Biol. Chem. **262**: 10863-10872; 1987.

119. Coll, J.M.; Hickey, R.J.; Wei, Y.; Malkas, L.H. The human cell multiprotein DNA replication complex (MRC): the effect of camptothecin on its ability to support *in vitro* DNA synthesis. CCP **39**: 97-102; 1996.

120. Wills, P.; Hickey, R.J.; Ross, D.; Cuddy, D.; Malkas, L.H. A novel *in vitro* model system for studying the action of ara-c. CCP **38**: 366-372; 1996.

121. Ross, D.D.; Cuddy, D.P.; Cohen, N.; Hensley, D.R. Mechanistic implication of alterations in HL-60 cell nascent DNA after exposure to 1-beta-D-arabinofuranosylcytosine. CCP **31**: 61-70; 1992.

122. Graham, F.L.; Whitmore, G.F. Studies in mouse L-cells on the incorporation of 1-beta-D-arabinofuranosylcytosine into DNA and on the inhibition of DNA polymerase by 1-beta-D-arabinofuranosylcytosine 5' triphosphate. Cancer Res. **30**: 2636-2644; 1970.

123. Graham, F.L.; Whitmore, G.F. The effect of 1-beta-D-arabinofuranosylcytosine on growth, viability and DNA synthesis of mouse L cells. Cancer Res. **30**: 2627-2635; 1970.

124. Magnusson, G.; Craig, R.; Narkhammar, M.; Reichard, P.; Staub, M.; Warner, H. Replication of polyoma DNA: effects of hydroxyurea and arabinosylcytosine. Cold Spring Harb. Symp. Quant. Biol. **39** (Pt 1): 227-233; 1975.

125. Malkas, L.H. SV40 in vitro DNA replication: a potential model for studying anticancer drug action. In: Snapka, R.M. (ed.) "The SV40 Replicon Model for Analysis of Anticancer Drugs" pp. 149-166, R.G. Landes Co., 1996.

126. Abrams, J.S.; Moore, T.D.; Friedman, M. New chemotherapeutic agents for breast cancer. Cancer **74**: 1164-1176; 1994.

127. Pfeiffer, P.; Cold, S.; Soren, C. Cytotoxic treatment of metastatic breast cancer. Which drugs and drug combinations to use? Acta Oncol. **31**: 219-224; 1992.

128. Vahdat, L.; Raptis, G.; Fennelly, D.; Crown, J. High-dose chemotherapy of metastatic breast cancer: a review. Cancer Invest. **13**: 505-510; 1995.

Chapter 2

Citation: Oncology Res. **8**: 435-447; 1996

The Human Breast Cell DNA Synthesome: Its Purification from Tumor Tissue and Cell Culture

Jennifer M. Coll [(a)], Jennifer W. Sekowski [(b,f)], Robert J. Hickey [(b-d,f)], Lauren Schnaper [(e,i)], Wei Yue [(a)], Angela M.H. Brodie [(a,c)], Lahja Uitto[(g)], Juhani Syvaoja [(g)] and Linda H. Malkas [(a-d,h)*]

[a]Department of Pharmacology and Experimental Therapeutics, [b]Program in Molecular and Cellular Biology, [c]Program in Oncology, [d]Program in Toxicology, [e]Department of Surgery, University of Maryland School of Medicine, Baltimore, MD 21201; [f]Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, MD 21201; [g]Biocenter Oulu and Department of Biochemistry, University of Oulu, FIN-90570 Oulu, Finland;

Running Title: The Breast Cell DNA Synthesome

Key Words: breast cancer; DNA replication; multiprotein complex; fidelity; DNA polymerase

ABSTRACT

In this report, we describe for the first time the isolation and purification of an organized multiprotein complex for DNA replication from MDA MB-468 human breast cancer cells. This complex, which we designate the DNA synthesome, fully supports the *in vitro* replication of simian virus 40 (SV40) origin-containing DNA in the presence of the viral large T-antigen. Since the SV40 virus utilizes the host's cellular proteins for its own DNA replication, our results indicate that the DNA synthesome may play a role not only in viral DNA synthesis but in human breast cell DNA replication as well. Our studies demonstrate that the following DNA replication proteins constitute the DNA synthesome: DNA polymerase α, DNA primase, DNA polymerase δ, proliferating cell nuclear antigen (PCNA), replication protein A (RP-A), replication factor C (RF-C), DNA topoisomerases I, II, and DNA polymerase ε. In addition, we successfully isolated the DNA synthesome from human breast tumor tissue as well as from xenografts from nude mice injected with the human breast cancer cell line MCF-7. The DNA synthesome purified from the breast cancer tissues fully supports SV40 DNA replication *in vitro*. Furthermore, our results obtained from a novel forward mutagenesis assay suggest that the DNA synthesome isolated from a non-malignant breast cell line mediates SV40 DNA replication by an error-resistant mechanism. In contrast, the DNA synthesome derived from malignant breast cells and tissue exhibited a lower fidelity for DNA synthesis *in vitro*. Overall, our data support the role of the DNA synthesome as mediating breast cell DNA replication.

Title Footnote:

* This work was supported in part by research awards CA57350 and CA65754 from the National Institutes of Health/National Cancer Institute to LHM. Also a University of Maryland School of Medicine Designated Research Initiation Fund award to LHM and a University of Maryland Cancer Center Research Award to RJH supported this work. JMC and JWS are recipients of U.S. Army Breast Cancer Research Program Predoctoral Fellowships.

h **Author: Linda H. Malkas**, author to whom correspondence should be addressed

Address: University of Maryland School of Medicine
(a) Department of Pharmacology and Experimental Therapeutics
(b) Program in Molecular and Cellular Biology
(c) Program in Oncology
(d) Program in Toxicology
655 W. Baltimore St.
Baltimore, MD 21201

Tel: (410) 706-2313 or 1798
Fax: (410) 706-0032

Author: Jennifer M. Coll

Address: University of Maryland School of Medicine
(a) Department of Pharmacology and Experimental Therapeutics

Author: Jennifer W. Sekowski

Address: University of Maryland School of Pharmacy
(f) Department of Pharmaceutical Sciences
(b) Program in Molecular and Cellular Biology

Author: Robert J. Hickey

Address: University of Maryland School of Pharmacy
(f) Department of Pharmaceutical Sciences
(b) Program in Molecular and Cellular Biology
(c) Program in Oncology
(d) Program in Toxicology

Author: Lauren Schnaper

Address: University of Maryland School of Medicine
(e) Department of Surgery

Present Address
(i) Breast Evaluation and Treatment Center
Greater Baltimore Medical Center
Suite 5140
Baltimore, MD 21240

Author: Wei Yue

Address: University of Maryland School of Medicine
(a) Department of Pharmacology and Experimental Therapeutics

Author: Angela M.H. Brodie

Address: University of Maryland School of Medicine
(a) Department of Pharmacology and Experimental Therapeutics
(c) Program in Oncology

Author: Lahja Uitto

Address: University of Oulu
(g) Biocenter Oulu and Department of Biochemistry

Author: Juhani Syvaoja

Address: University of Oulu
(g) Biocenter Oulu and Department of Biochemistry

Abbreviations used:

SV40, simian virus 40; PCNA, proliferating cell nuclear antigen; RP-A, replication protein A; RF-C, replication factor C; PMSF, phenylmethyl sulfonyl fluoride; AAN, aminoacetonitrile hemisulfate; TDEG, 50 mM Tris-HCl, pH 7.5, 1mM DTT, 1mM Na_3EDTA, 10% glycerol; NE, nuclear extract; IPTG, isopropyl B-D-thiogalactopyranoside.

INTRODUCTION

Breast cancer is one of the most commonly diagnosed female cancers and the second leading cause of cancer death among women [1]. Recently, numerous reports have underscored the important role of cell proliferation rate as a prognostic factor for breast carcinoma. Studies using flow cytometry to measure the DNA content of breast tumor cells show a strong association between a high S-phase fraction and poor prognosis for relapse-free survival in patients with lymph node negative breast cancer [2]. Additionally, mammary cancer cells exhibit extensive DNA damage [3], as compared with non-malignant breast cells. The increased mutation frequency that accompanies the cellular transformation process is postulated to arise from molecular alterations of specific DNA replication and/or repair proteins [4]. Despite the knowledge that a high proliferation activity and increased mutation frequency correlate with breast cancer progression, there is a paucity of information regarding the regulation and precise molecular mechanisms of human breast cell DNA replication.

To date, several mammalian enzymes and proteins have been shown to be required for DNA replication *in vitro* [5-10]. In particular, the proteins necessary to support SV40 based cell-free DNA synthesis include: DNA polymerase α, DNA primase, DNA polymerase δ, proliferating cell nuclear antigen (PCNA), replication protein A (RP-A), replication factor C (RF-C), and DNA topoisomerases I and II [11]. As mammalian cell DNA replication represents an intricate yet highly coordinated and efficient process, it follows that the proteins mediating DNA synthesis may be organized into a multiprotein complex. In support of this hypothesis, several reports have described the

isolation of large macromolecular complexes of replication essential proteins from extracts of eukaryotic cells [9,11,12].

Our laboratory was the first to isolate and characterize a multiprotein DNA replication complex from human (HeLa) cells and murine (FM3A) mammary carcinoma cells that fully supports origin specific and large T-antigen dependent papovavirus DNA replication *in vitro* [13-15]. The DNA products synthesized by the human cell multiprotein complex consist of monomeric circular form I and II DNAs as well as topological and replicative intermediates [13]. Furthermore, the majority (80-90%) of these products are resistant to *DpnI* digestion, which is consistent with the criteria for semi-conservative replication of full-length DNA [13,15].The multiprotein complex was observed to retain its ability to replicate papovavirus DNA after additional purification by anion-exchange chromatography and sucrose or glycerol gradient sedimentation [13-15]. In addition, the integrity of the multiprotein complex was maintained after its treatment with salt, detergents, RNase, DNase and electrophoresis through native polyacrylamide gels [15,16]. These results suggest that the association of the proteins with one another is independent of non-specific interactions with other cellular macromolecules.

We report here, for the first time, that breast cancer cells also utilize a multiprotein complex to carry out cellular DNA synthesis, and we now designate this complex the DNA synthesome. We describe the isolation and purification of the DNA synthesome from human (MDA MB-468) breast cancer cells and most importantly from human breast tumor cell xenografts, as well as from biopsied human breast tumor tissue. Furthermore, we discuss the results of a novel forward mutagenesis assay which establish that the DNA synthesome isolated from breast cancer cells and tissue has a comparatively

lower fidelity for DNA replication than the synthesome isolated from a normal breast cell line. Ultimately, we anticipate that the complete characterization of this DNA synthesome will lead to important new insights into understanding the molecular mechanisms of breast cancer cell DNA replication.

Materials and Methods

Materials. [α-^{32}P]dCTP (3000 Ci/mmol; 370 MBq/ml; 10 mCi/ml) and [^{3}H]thymidine (90 Ci/mmol; 37 MBq; 2.5 mCi/ml) were obtained from DuPont New England Nuclear (Boston, MA). Camptothecin was purchased from TopoGen, Inc. (Columbus, Ohio). The drug was dissolved in dimethyl sulfoxide and stored in aliquots at -20°C. Purified topoisomerase I enzyme (4 ng/ml) and a topoisomerase II assay kit were purchased from TopoGen, Inc. (Columbus, Ohio).

Cell Culture. Suspension cultures of MDA MB-468 human breast cells were adapted from monolayer cultures. The cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of irradiated new-born calf serum and FBS. Exponentially growing cells ($5x10^5$ cells/ml medium) were harvested and washed three times with PBS: 20 mM Na_2HPO_4, 0.15 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4. The cells were then pelleted by low-speed centrifugation (1000rpm, 5 minutes, 4°C), and the cell pellets stored at -80°C until fractionation. Hs587Bst cells were cultured in monolayer in DMEM supplemented with 30 ng/ml of epidermal growth factor and 10% FBS. Sub-confluent cells were harvested and washed three times with PBS. The cells were then pelleted by low-speed centrifugation (1000 rpm, 5 minutes, 4°C) and the pellets stored at -80°C until fractionation. MCF-7 cells were cultured in Eagle's minimum essential medium containing 5% FBS and 600 μg/ml of neomycin sulfate, as described in Yue et al. [17]. Subconfluent MCF-7 cells were scraped into Hank's solution and centrifuged at 1000 rpm for 2 minutes at 4°C. The cells were then prepared for inoculation into intact nude mice according to published procedures [17].

Human Breast Tumor Tissue. A biopsy from an infiltrating ductal type carcinoma of the female mammary gland was immediately frozen at -80°C after resection. To examine the tumor tissue for the presence of a functional DNA synthesome, the breast tumor tissue was thawed and subjected to the purification protocol described in a later section of these Materials and Methods.

Isolation and Purification of the DNA Synthesome from Breast Cancer Cells: Cell Fractionation. MDA MB-468 (20 g) and Hs587Bst (2 g) cells were homogenized and the breast cell DNA synthesome was purified according to our previously published procedures [13-15] and as outlined in Figure 1. Briefly, the respective cell pellet was resuspended in 2 volumes of buffer (50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 5 mM $MgCl_2$, 0.1 mM PMSF, 0.1 mM AAN (pH 7.5) and 1 mM DTT) and homogenized using a loose-fitting Dounce homogenizer. The homogenate was then fractionated into a nuclear pellet and cytosolic extract. The nuclei were extracted with a low salt buffer (0.15 M KCl) while the cytosolic fraction was used to prepare a post-microsomal supernatant (S-3). The nuclear extract and the post-microsomal supernatant were combined and adjusted to 2M KCl and 5% (w/v) polyethylene glycol. The mixture was rocked for 1 h at 4°C, then centrifuged at 16,000 rpm for 15 minutes (4°C). The resulting supernatant was then dialyzed against buffer A [13] containing 0.25 M sucrose. The dialyzed fraction was clarified by centrifugation at 16,000 rpm for 15 minutes and the supernatant solution was layered onto a 1 ml 2 M sucrose cushion containing buffer A. After centrifugation at 40,000 rpm for 16 h (4°C), the supernatant S-4 and sucrose interface P-4 fractions were collected and dialyzed against buffer B [13]. The fractions were then immediately tested for DNA polymerase α and *in vitro* SV40 DNA replication activities.

Column Chromatography. Five milliliters of the dialyzed MDA MB-468 P-4 fraction were loaded onto a 1 ml Q-Sepharose (Pharmacia, Piscataway, NJ) column (1 cm^3 bed volume/25 mg protein) pre-equilibrated with buffer B. The protein not binding to the matrix was collected and designated the column flow-through. After washing the matrix with 8 column volumes of buffer B, the column was eluted with 10 volumes of a linear 50-500 mM gradient of KCl. Fractions of 0.4 ml were collected and assayed for protein and enzymatic activity. Fractions containing the peak of DNA polymerase α and *in vitro* SV40 DNA replication activities were pooled, dialyzed against TDEG buffer [13] and stored at -80°C.

Velocity Sedimentation Analysis of the DNA Synthesome Isolated from MDA MB-468 Breast Cancer Cells. Six hundred microliters (600 μg of protein) of the DNA synthesome present in the Q-Sepharose peak fraction was layered over a 10 ml 10-30% sucrose gradient containing 0.5 M KCl. Velocity sedimentation analysis was performed as described in a previously published report from this laboratory [15]. The sedimentation analysis of marker proteins (horse spleen apoferritin (17S) and yeast alcohol dehydrogenase (7S)) was performed on parallel gradients to verify that the gradient was isokinetic.

Micro-isolation and Purification of the DNA Synthesome from Breast Tumor Tissue: **Cell Fractionation**. The DNA synthesome was purified from breast cancer tissue according to a modified version of the isolation scheme depicted in Figure 1 and as described in a previous section of these methods. All steps of the fractionation process were altered to facilitate the purification of the DNA synthesome from small quantities of breast tumor tissue. The human breast tumor (8.55 g) was dissected and finely chopped

with a tissue chopper at 4°C. The minced breast tissue was then suspended in 1 volume of buffer (50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 5 mM $MgCl_2$, 0.1 mM PMSF, 0.1 mM AAN (pH 7.5) and 1 mM DTT) and homogenized using a 1 ml Dounce homogenizer. The homogenate was centrifuged at 2,000 rpm for 10 minutes (4°C), and the crude nuclear pellet (NP) and cytosolic fraction (S-1) were collected separately. The nuclear pellet was resuspended in 1 volume of nuclei extraction buffer [13] containing 0.15 M KCl. After rocking the nuclear pellet for 2 h at 4°C, the extracted nuclei were centrifuged at 22,300 rpm for 2.1 minutes (4°C) using a TLA 100.3 rotor and the supernatant (NE) retained. To remove mitochondria and microsomes, the S-1 fraction was subjected to differential centrifugation using a TLA 100.3 rotor: 17,800 rpm, 3.2 minutes and 59,700 rpm, 22.2 minutes, respectively. The final post-microsomal supernatant (S-3) was collected. The NE was combined with the S-3 fraction; 4.5 ml of the NE/S-3 fraction was then layered over a 0.5 ml 2 M sucrose cushion. After centrifugation at 40,000 rpm for 16h (4°C) using a Beckman (Columbia, MD) SW55.Ti swinging bucket rotor, the S-4 and P-4 fractions were collected, dialyzed against a low-salt TDEG buffer, and assayed for their respective enzymatic activities. These same steps were followed to purify the DNA synthesome from the xenografts grown in nude mice.

Column Chromatography. Seven hundred microliters of the dialyzed human breast tumor P-4 fraction (3.3 mg protein) were loaded onto a 0.15 ml DE52 cellulose column, pre-equilibrated in TDEG buffer containing 5 mM KCl. The protein not binding to the matrix was collected, and designated the column flow-through. The column was washed with 8 column volumes of pre-equilibration buffer. Matrix-bound protein was then eluted with 8 column volumes of TDEG buffer containing 1M KCl. Fractions of approximately

0.1 ml were collected, dialyzed against TDEG buffer, and assayed for their DNA polymerase α and *in vitro* DNA replication activities.

Purification of SV40 Large T-antigen. SV40 large T-antigen was purified from 293 cells infected with a recombinant adenovirus vector, Ad-SVR284, as detailed elsewhere [18].

***In vitro* SV40 DNA Replication Assay.** Assay reaction mixtures (12.5 µl) contained 80 mM Tris-HCl (pH 7.5); 7 mM $MgCl_2$; 1 mM DTT; 3-20 µg protein fraction; 0.5-1.0 µg purified SV40 large T-antigen; 25 ng plasmid $pSVO^+$ [19] containing and insert of SV40 replication-origin DNA sequences; 100 µM each dTTP, dATP, dGTP; 200 µM each rCTP, rGTP, UTP; 4 mM ATP; 25 µM [α-^{32}P]dCTP; 40 mM creatine phosphate; 1µg creatine kinase. Each reaction was incubated for 2h at 35°C. The replication assay reaction products were processed using DE81 (Whatman) filter binding to quantitate the amount of radiolabel incorporated into the replication products [20]. One unit of SV40 replication activity is equivalent to the incorporation of 1 pmol dCMP into SV40 origin-containing plasmid DNA per 2h under these described assay conditions.

Enzyme Assays. DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures [21,22]. One unit of DNA polymerase α activity is equivalent to 1nmol of total [^{3}H]TMP incorporated into DNA per hour at 35°C. The assay for **DNA topoisomerase I** activity is a modification of published methods [19] and is described in detail by Hickey et al. [23]. **DNA topoisomerase II** activity was measured using an assay kit purchased from TopoGen, Inc.

Immunodetection of DNA Polymerases δ, ε, RP-A, RF-C, PCNA and DNA Primase. Denaturing polyacrylamide gel electrophoresis of the various protein fractions was performed as previously described [24]. The resolved polypeptides were transferred (20 volts, 16h, 4°C) to nitrocellulose membranes, and immunodetection of the respective DNA replication proteins was performed using a light-enhanced chemiluminescence system (Amersham). A monoclonal antibody prepared against the C-terminal portion of DNA polymerase δ was used at a 1:100 dilution to probe membranes for the 125 kDa polymerase δ polypeptide. The anti-polymerase ε antibody which recognizes the 140- and >200 kDa forms of polymerase ε, was used at a 1:1000 dilution. Both the anti-RF-C monoclonal antibody (mAb-11), which recognizes the 140 kDa subunit of the RF-C protein-complex, and the anti-RP-A antibody (p34-20), which recognizes the 34 kDa subunit of RP-A, were generous gifts from Dr. Bruce Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Both antibodies were used at a 1:500 dilution. The anti-DNA primase antibody, a gift from Dr. William Copeland (National Institute of Environmental Health Sciences, Research Triangle Park, NC), was used at a 1:500 dilution. The anti-PCNA antibody was used at a dilution of 1:1000. The appropriate species-specific horseradish peroxidase conjugated secondary antibodies were used in the immunoblots at a dilution of 1:5000.

Forward Mutagenesis Assay: Transfection and Plating. Two hundred nanograms of pBK-CMV plasmid DNA (Stratagene, LaJolla, CA), encoding the *lac-Zα* gene, was incubated with 35-45 μg of protein fraction per *in vitro* DNA replication assay. The replicated pBK-CMV DNA was then *Dpn*I digested, precipitated as described [20] and used in the transfection of *Escherichia coli* strain XL1-Blue MRF'(Stratagene):

[(mcrA) 183, δ(mcr(B-hsdSMR-mrr) 173, endA1, supE44, thi_1, recA1, gyrA96, relA1, lac[F'proAB, lac1^{q}Z(m15, Tn10(tet^{R})]. Forty microliters of bacterial stocks maintained in Luria broth, containing 10% glycerol, were mixed with 300 pg of the pBK-CMV DNA replicated *in vitro*, and this mixture was incubated for 10 minutes on ice; subsequently, the DNA was electroporated into the cells under the following conditions: 1.4 kV, 25 μF, 200 ohms. Immediately following electroporation, 960 μl chilled, sterile SOC buffer (20 mM glucose in Luria broth medium) was added to the reaction cuvette. The electroporated mixture was then incubated in a rotary shaker (250 rpm) at 35°C for 1h. An aliquot of the incubated culture, sufficient to yield 100-600 bacterial colonies per plate, was spread on top of 20 ml solidified Luria broth agar containing 0.5 mg/ml kanamycin, 25 mg/ml IPTG and 25 mg/ml X-gal. (These plating conditions yield an intense blue colored bacterial colony when the bacteria express the unmutated plasmid and a light blue to white colored bacterial colony when the bacteria contain plasmids harboring mutations in the *lac-Zα* gene.) As a negative control for the forward mutagenesis assay, $2x10^9$ bacterial cells were transfected with 200 ng of unreplicated, *Dpn*I digested pBK-CMV DNA; virtually no antibiotic-resistant colonies were produced under these conditions (data not shown). Also, the background mutation frequency for this assay was determined by transfecting 10^9 bacterial cells with 200 ng of unreplicated pBK-CMV DNA. Only one mutant colony per 10^6 wild-type colonies was produced under these conditions (data not shown).

Scoring of Mutant Phenotypes. Mutant phenotypes, resulting from the inactivation of the *lac-Zα* gene in the pBK-CMV plasmid, were scored after approximately 12-15 hours of incubation at 35°C by a modification of a procedure described by the laboratory of

Kunkel [25]. To score the variable color intensities of the mutant phenotypes reproducibly, a scale of blue color intensities has been established [25]. Unmutated pBK-CMV DNA generates a dark blue color which, on a scale of 0-4, is assigned a value of 4. The variable mutant phenotypes are distinguished as 0^+(white/colorless), 1^+(faint blue), 2^+(medium blue) and 3^+(almost wild-type). To eliminate false positives resulting from plating artifacts, mutant colonies were picked from the plates, diluted in 50 mM sodium borate buffer (pH 9.0) and mixed with an equal dilution of bacteria containing unreplicated pBK-CMV plasmid. Plating of this mixture on the agar plates containing the color substrate X-gal (see above) enhances the contrast between the wild-type and mutant phenotypes as well as permits the scoring of subtle phenotypic differences arising from small variations in the position and number of point mutations within the *lac-Zα* gene.

RESULTS

Human Breast Cancer Cell DNA Replication Proteins Co-fractionate as a Readily Sedimentable Form.

To determine whether a sedimentable complex of DNA replication proteins could be isolated from human breast cancer cells, as previously demonstrated for HeLa [13,14] and FM3A cells [15], we subjected MDA MB-468 cells to the fractionation scheme outlined in Figure 1. The PEG NE/S-3, S-4 and P-4 fractions were collected and assayed for DNA polymerase α activity. The majority of the enzyme's activity partitioned with the sedimentable P-4 fraction following polyethylene glycol precipitation and discontinuous gradient centrifugation of the NE/S-3 fraction (Table 1). This result is consistent with our earlier work on the purification of the DNA synthesome from HeLa and FM3A cells [13-15], in which the DNA polymerase α activity contained in the DNA synthesome partitioned to the P-4 fraction at the sucrose interface.

In addition to determining DNA polymerase α activity, we assayed the PEG NE/S-3, S-4 and P-4 fractions for *in vitro* SV40 DNA replication activity (Materials and Methods). DE81 filter binding analysis was used to quantitate the level of [α-^{32}P]dCMP incorporation into SV40 DNA replication products. Following subfractionation of the PEG NE/S-3 fraction into the S-4 and P-4 fractions, the ability to support SV40 DNA replication *in vitro* partitioned exclusively with the sedimentable P-4 fraction (Table 1). This pattern of partitioning of DNA replication activity is also consistent with our earlier work on the purification of the synthesome from HeLa and FM3A cells [13-15]. Only negligible amounts of radiolabel were incorporated into DNA replication products when reactions lacked SV40 large T-antigen. These data indicate that all of the activities

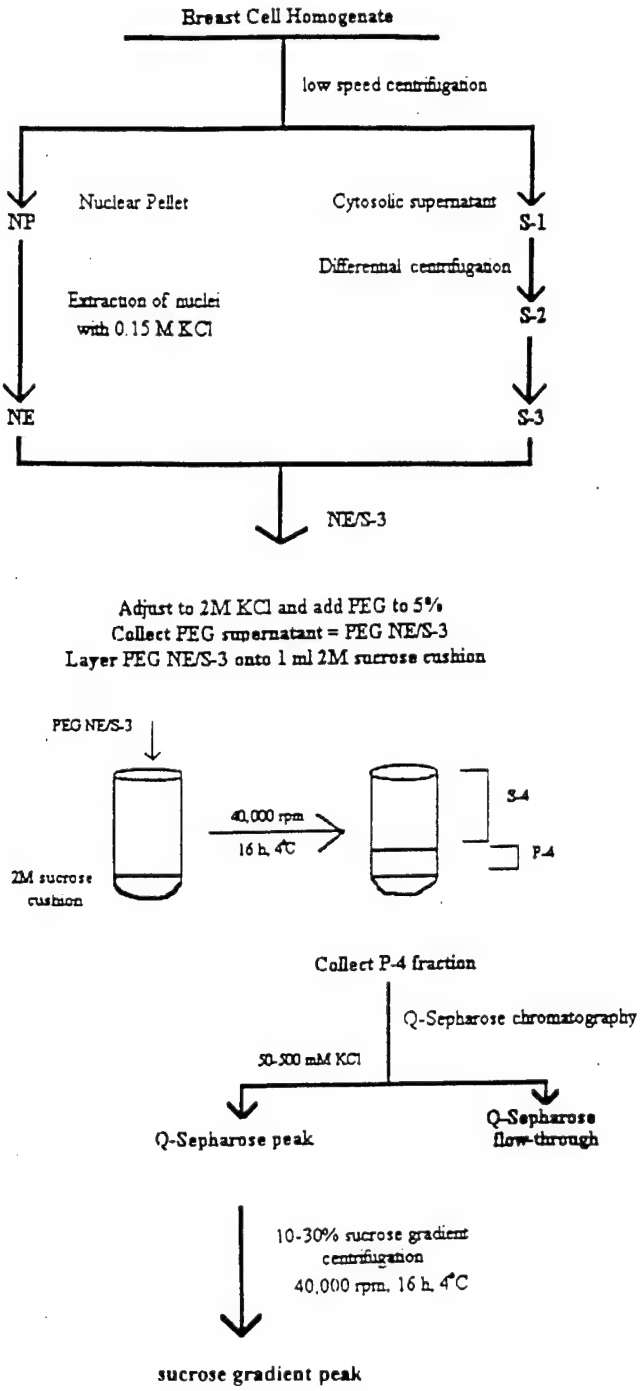


Figure 1. Flow diagram of the isolation scheme used to purify the DNA synthesome from MDA MB-468 human breast cancer cells. A detailed description of the isolation scheme is presented in the Materials and Methods.

Table 1. DNA polymerase α and *in vitro* DNA replication activities of the DNA synthesome from MDA MB 468 human breast cancer cells.

FRACTION	PEG NE/S-3	S-4	P-4	QS Peak	FT	SG
DNA polymerase α[a]	132.5	0.3	188.3	N.D.[c]	1.01	N.D.[c]
DNA replication (+T)[b]	103.8	8.8	110.6	136.6	3.4	141.2
DNA replication (-T)[b]	3.1	0.0	0.2	8.5	1.8	10.2

[a]DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures. One unit of DNA polymerase activity is equivalent to $1x10^{-10}$ mol of ^{3}[H]-TMP incorporated into DNA per hour at 35°C. These values represent the average of three independent experiments.

[b]*In vitro* SV40 DNA replication assays were performed as described previously. One unit of replication activity equals the incorporation of 1 pmol of ^{32}[P]-dCMP into SV40 origin containing DNA. These values represent the average of three independent experiments.

[c]Not determined.

required to execute large T-antigen dependent SV40 DNA replication reside in the human breast cancer cell sedimentable P-4 fraction.

Further Purification of the Human Breast Cancer Cell DNA Synthesome.

We further purified the breast cancer cell DNA synthesome from the sedimentable P-4 fraction by Q-Sepharose anion-exchange chromatography, a method successfully employed for the purification of the DNA synthesome from HeLa cells [13,14]. The P-4 fraction was applied to a 1-ml Q-Sepharose column and the DNA synthesome eluted by a linear gradient of KCl (50-500 mM). Figure 2 shows the profile of DNA polymerase α activity as it eluted from the Q-Sepharose column. The DNA polymerase α activity eluted from the column as an initial sharp peak at lower salt concentrations (fractions 6-10), with an additional small peak of activity at higher salt concentrations (fractions 21-23). Negligible amounts of enzyme activity were found in the column flow-through and wash fractions (data not shown).

The peak of DNA polymerase α activity that eluted from the Q-Sepharose column (fractions 6-10) was designated the Q-Sepharose peak. Both the peak and the column flow-through fractions were assayed for *in vitro* SV40 DNA replication activity. The Q-Sepharose peak contained over 80% of the large T-antigen dependent *in vitro* DNA replication activity; the column flow-through fraction supported significantly less DNA synthesis (Table 1).

Velocity Sedimentation Analysis of the Breast Cancer Cell DNA Synthesome.

We determined the sedimentation coefficient of the breast cancer cell DNA synthesome by subjecting the Q-Sepharose peak fraction to velocity sedimentation analysis in a 10-30% sucrose gradient containing 0.5M KCl [15]. The sucrose gradient

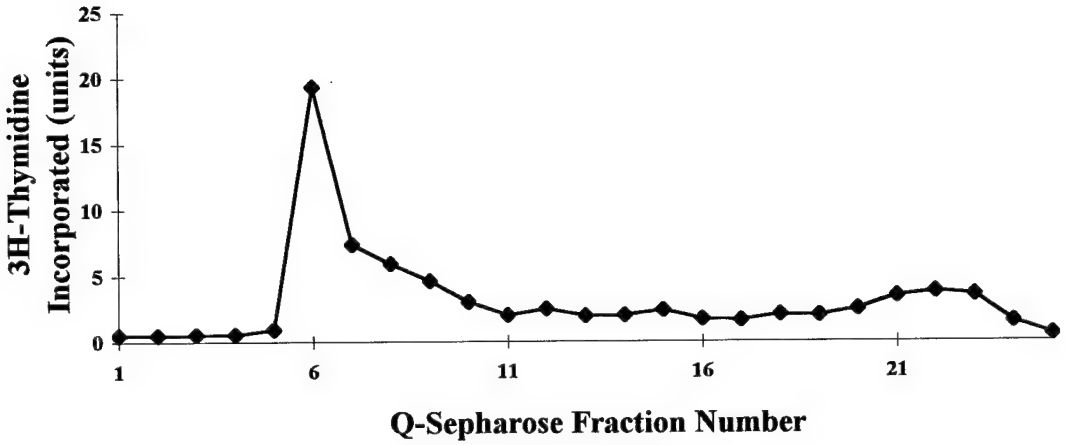


Figure 2. Q-Sepharose chromatographic profile of DNA polymerase α activity in the MDA MB-468 derived P-4 fraction. A description of the column preparation and elution conditions are provided in the text and Materials and Methods.

fractions were collected and assayed for DNA polymerase α and *in vitro* SV40 DNA replication activities. Both activities co-sedimented in the sucrose gradient with a sedimentation coefficient of 18S (Figure 3; Table 1). This 18S sedimentation coefficient for the breast cell DNA synthesome corresponds to the S-value obtained for the HeLa cell DNA synthesome. Presumably, the 18S value of the human breast cancer cell DNA synthesome accounts for its ready sedimentation to the sucrose interface following the centrifugation of the PEG NE/S-3 fraction (Figure 1).

The DNA Replication Proteins that Copurify with the Breast Cancer Cell DNA Synthesome.

We performed Western blot analyses and enzyme assays to identify the DNA replication proteins that copurify with the breast cancer cell DNA synthesome during its various stages of purification. As numerous studies have shown that DNA polymerase δ plays an integral role in the *in vitro* synthesis of SV40 origin containing DNA [8,26,27], we probed the PEG NE/S-3, P-4, S-4, Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions for the presence of the protein. Utilizing a monoclonal antibody prepared against the C-terminal peptide of DNA polymerase δ [28], we found that the protein exclusively co-purified with the replication-competent P-4, Q-Sepharose peak and sucrose gradient peak fractions (Figure 4). The enzyme was not detectable in the replication-deficient S-4 and Q-Sepharose flow-through fractions.

In addition to DNA polymerase δ, we examined the human breast cancer cell fractions for the presence of RF-C [27,29] and DNA primase [27]. Immunoblot analyses, using antibodies that recognize either the 140 kDa subunit of the RF-C protein complex

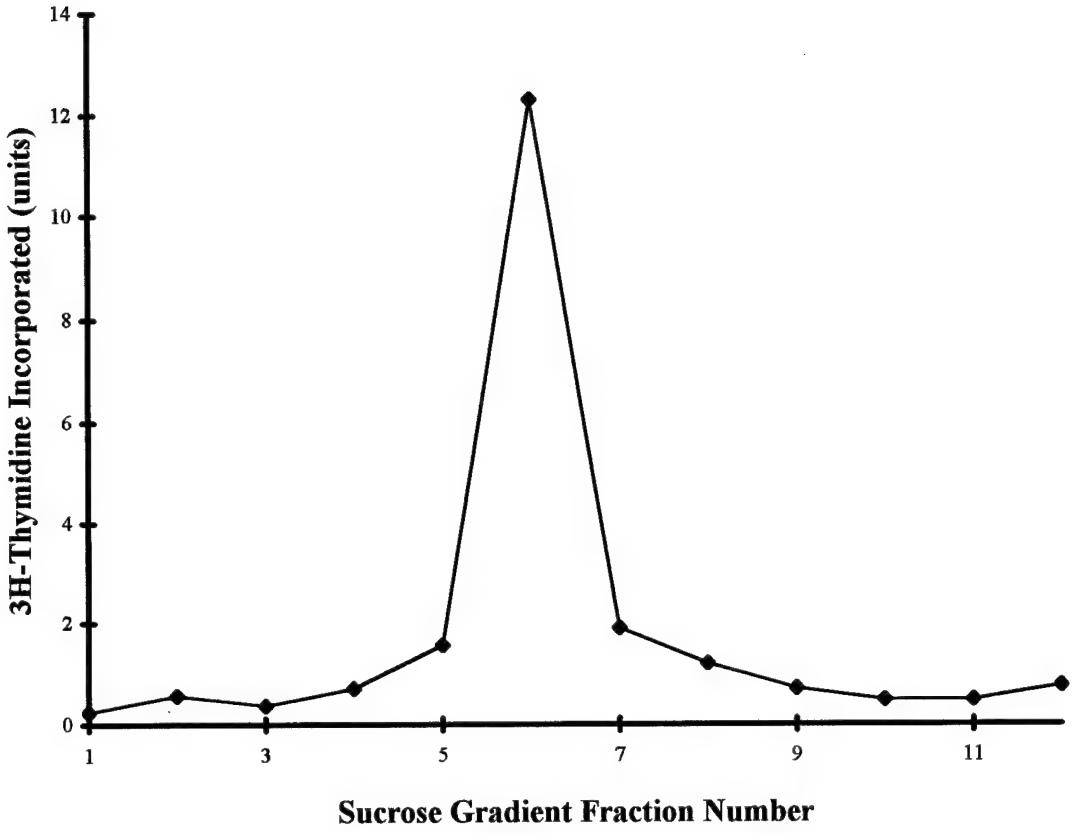


Figure 3. Velocity sedimentation analysis of the DNA synthesome present in the MDA MB-468 Q-Sepharose peak fraction. Eight hundred microliters of the Q-Sepharose peak fraction was layered onto a 9 ml 10-30% sucrose gradient containing 0.5 M KCl. Centrifugation was performed as described in the Materials and Methods. The assay for DNA polymerase α activity was performed according to published procedures [21,22]; one unit of activity denotes 1 nmol TMP incorporated into DNA per hour at 35°C.

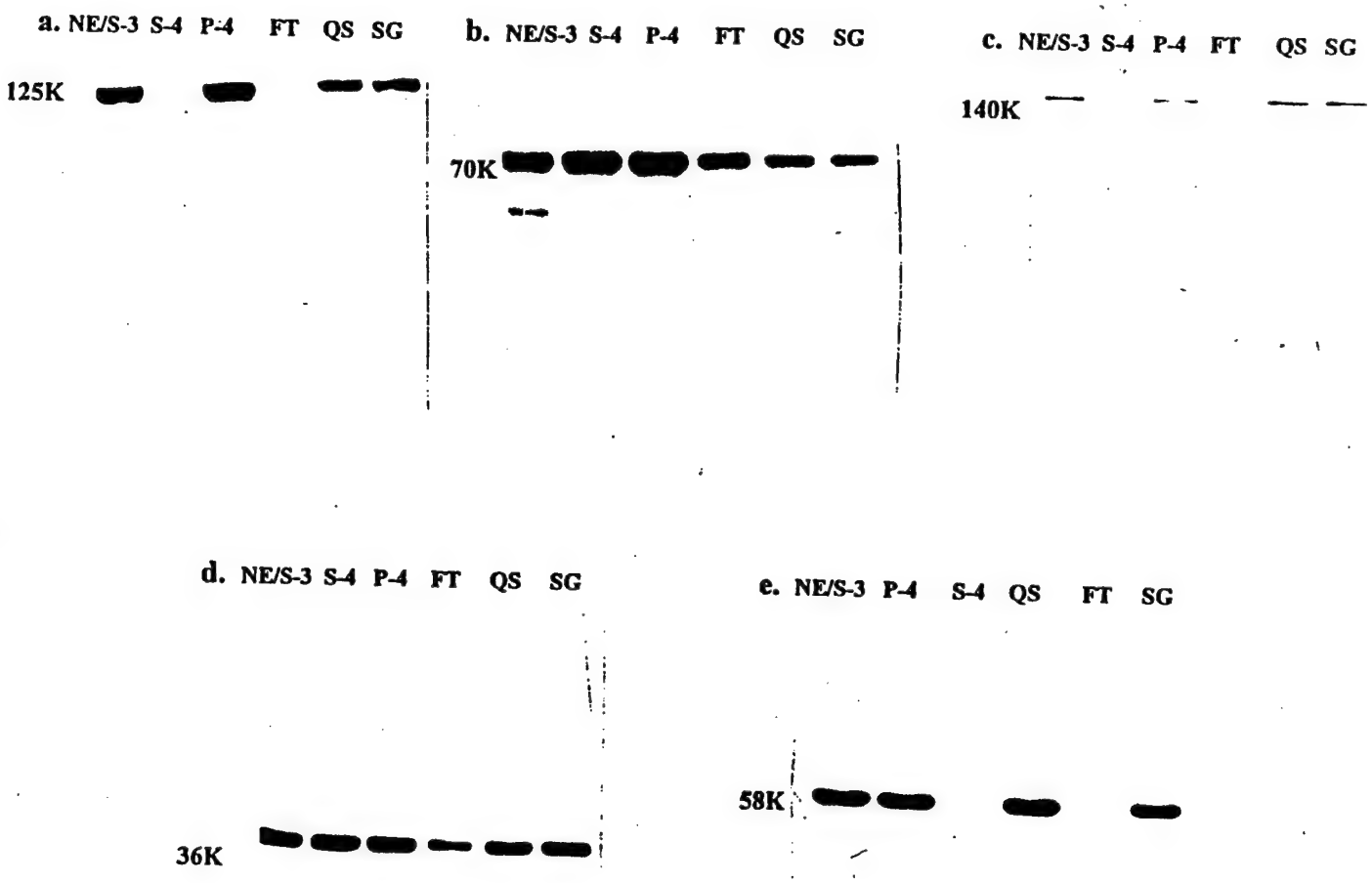


Figure 4. Western blot analysis of the MDA MB-468 breast cancer cell derived fractions. Thirty micrograms of each protein fraction (PEG NE/S-3, P-4, S-4, Q-Sepharose peak (QS), Q-Sepharose flow-through (FT) and sucrose gradient peak (SG)) was resolved on 8% polyacrylamide gels, then transferred to nitrocellulose membrane filters. The membranes were incubated with primary antibodies against (a) DNA polymerase δ, (b) RP-A, (c) RF-C, (d) PCNA and (e) DNA primase. Following incubation with the appropriate species-specific secondary antibody conjugated to horseradish peroxidase, the immobilized proteins were detected using a light-enhanced chemiluminescence system (Amersham).

or the 58 kDa subunit of DNA primase, revealed that RF-C and DNA primase resided only in the replication-competent protein fractions (Figure 4).

Western blot analysis also shows that the DNA replication protein PCNA was present in the replication-competent breast cancer cell fractions, as well as the S-4 and Q-Sepharose flow-through fractions (Figure 4). This result suggests that PCNA may not be as tightly associated with the DNA synthesome as compared to DNA polymerases α, δ, RF-C and DNA primase. Furthermore, immunodetection of RP-A [30,31] with a monoclonal antibody to the 70 kDa subunit of the protein, reveals that the polypeptide fractionated with both the replication-competent and -deficient fractions (Figure 4). These results suggest that only a fraction of the cellular pools of PCNA and RP-A copurify with the breast cancer cell DNA synthesome.

Furthermore, we determined whether the breast cancer cell DNA synthesome possesses DNA topoisomerase I activity by assaying several breast cancer fractions for the respective enzymatic activity (Materials and Methods). In Figure 5, lanes 1-3 show the conversion of supercoiled form I DNA to relaxed, open circular form II DNA by the topoisomerase I activity present in the Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions. Importantly, the relaxation of supercoiled plasmid DNA by the Q-Sepharose peak fraction was inhibited by 200 μM camptothecin (Figure 5, lane 4), a specific inhibitor of DNA topoisomerase I [32]. This indicates that the conversion of supercoiled plasmid DNA to form II DNA was mediated specifically by topoisomerase I.

As with PCNA, RP-A and DNA topoisomerase I, only a fraction of the total cellular pool of DNA topoisomerase II co-purifies with the breast cancer cell DNA synthesome. We assayed the Q-Sepharose peak, Q-Sepharose flow-through and sucrose

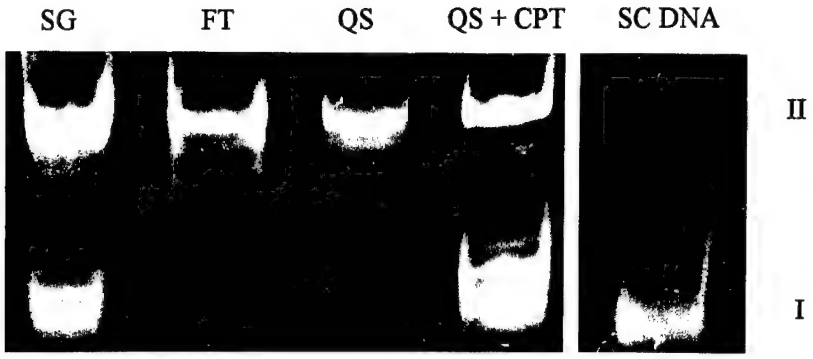


Figure 5. DNA topoisomerase I activity in the Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions. Reaction assays containing 8 μg of the Q-Sepharose peak (QS), 8 μg of the Q-Sepharose flow-through (FT) or 20 μg of the sucrose gradient peak (SG) were incubated for 30 minutes at 37°C with 0.3 μg of pSVO$^+$ plasmid DNA. Reactions were stopped by the addition of 1% SDS and topoisomers were resolved on a 1% agarose gel. After ethidium bromide (0.5 μg/ml) staining of gels, topoisomers were visualized with an ultraviolet light source. Lanes 1-3 show the conversion of supercoiled, form I DNA to relaxed, open circle form II DNA by the topoisomerase I activity present in the SG, FT and QS fractions, respectively. Lane 4 shows the inhibition of QS topoisomerase I activity by 200 μM of camptothecin [32]. Lane 5 shows the position of supercoiled plasmid pSVO$^+$.

gradient peak fractions for DNA topoisomerase II activity. The decatenation of interlocked aggregates of *Crithidia fasisculata* kinetoplast DNA to monomeric, open circular DNA by the topoisomerase II enzyme present in all three fractions is shown in Figure 6 (lanes 2-4). In addition, we determined that the Q-Sepharose peak, flow-through and sucrose gradient peak fractions were devoid of nuclease contamination because they did not support the relaxation of kinetoplast DNA to the linear DNA fragments (Figure 6). Moreover, as DNA topoisomerase II requires ATP for catalytic activity, incubation of the Q-Sepharose peak with a reaction buffer lacking ATP did not support the relaxation of kinetoplast DNA (Figure 6, lane 1).

Isolation of the DNA synthesome from Breast Tumor Tissue.

To verify that the DNA synthesome could be isolated from breast cancer tissue as well as from breast cancer cells, we subjected biopsied human breast tumor tissue to a modified version of the purification scheme depicted in Figure 1 (Materials and Methods). The alterations to the purification protocol were made to facilitate the isolation of the DNA synthesome from small quantities of breast tumor tissue. We collected and assayed the NE/S-3, P-4 and S-4 fractions for DNA polymerase α and large T-antigen dependent SV40 DNA replication activities. Table 2A shows that the majority of both activities partitioned exclusively with the sedimentable P-4 fraction after discontinuous gradient centrifugation of the NE/S-3 fraction.

We further purified the DNA synthesome that was isolated from the human breast tumor tissue using anion exchange chromatography (Materials and Methods). We collected and assayed the column fractions for both DNA polymerase α and *in vitro* SV40 DNA replication activities. A peak of DNA polymerase α activity (fractions 2,3)

Figure 6. DNA topoisomerase II activity in the Q-Sepharose peak, Q-Sepharose flow-through and sucrose gradient peak fractions. Decatenation reactions were performed in topoisomerase II buffer (TopoGen) with 0.15 μg kinetoplast DNA (KDNA) and 10μg of the respective protein fraction. Lane 1 shows the position of KDNA networks after incubation with Q-Sepharose peak (QS) in a buffer lacking ATP. Lanes 2-4 show the relaxation of KDNA to nicked, open circular DNA by the topoisomerase II activity present in the QS, flow-through (FT) and sucrose gradient peak (SG) fractions. Lane 5 shows the positions of the decatenated KDNA markers: nicked, open circular (top), linear (bottom). All reactions were stopped by the addition of a stop buffer containing 1% SDS. Reactions were loaded directly onto a 1% agarose gel containing 0.5 μg/ml ethidium bromide. After electrophoresis, DNA products were visualized with an ultraviolet light source.

Table 2A. DNA polymerase α and *in vitro* DNA replication activities of the DNA synthesome from human breast tumor tissue.

FRACTION	NE/S-3	S-4	P-4
DNA polymerase α[a]	27.8	1.7	37.5
DNA Replication +T[b]	29.5	1.8	122.9
DNA Replication -T[b]	5.74	0.5	12.1

[a]DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures. One unit of DNA polymerase activity is equivalent to $1x10^{-10}$ mol of ^{3}[H]-TMP incorporated into DNA per hour at 35°C. These values represent the average of two independent experiments.

[b]*In vitro* SV40 DNA replication assays were performed as described previously. One unit of replication activity equals the incorporation of 1 pmol of ^{32}[P]-dCMP into SV40 origin-containing DNA. These values represent the average of two independent experiments.

Table 2B. DNA polymerase α activity of the column purified DNA synthesome from human breast tumor tissue.

Fraction	Column Peak	Flow-through
DNA pol α[a]	77.3	1.3

[a]DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures. One unit of DNA polymerase activity is equivalent to $1x10^{-10}$ mol of ^{3}[H]-TMP incorporated into DNA per hour at 35°C. These values represent the average of two independent experiments.

was found to elute from the column in the presence of 1M KCl (Table 2B). In contrast, only a minor amount of DNA polymerase α activity was found in the column flow-through fraction (Table 2B). We also tested the fractions containing the peak polymerase α activity (fractions 2,3) as well as the column flow-through for *in vitro* SV40 DNA replication activity. Only fractions 2 and 3 supported SV40 DNA replication; the column flow-through did not contain DNA replication activity (data not shown).

We also fractionated breast cancer tissue-derived from a xenograft nude mouse model [17]. MCF-7 human breast cancer cells inoculated into nude mice develop into mammary tumors. The tumors were removed and homogenized. By using the modified purification protocol, it was found that most of the DNA polymerase α and DNA replication activities resided with the sedimentable P-4 fraction following discontinuous gradient centrifugation of the NE/S-3 fraction (Table 3). These results suggest that the DNA synthesome exists as a functional complex within intact human breast cancer cells.

DNA Polymerase ε Copurifies with the Breast Cancer Cell DNA Synthesome.

Several lines of evidence support a role for DNA polymerase ε in cellular DNA replication. First, DNA polymerase ε is more abundant in proliferating tissues than in non-proliferating tissues [33]. Second, when quiescent human fibroblast cells are stimulated to proliferate, the mRNA levels of DNA polymerase ε, like those of polymerase α, dramatically increase just prior to S-phase [33]. Third, when the gene encoding the yeast homologue of DNA polymerase ε is mutated, the yeast cells fail to proliferate, suggesting a critical role for this polymerase in cell proliferation [34]. To determine whether DNA polymerase ε copurifies with the breast cancer cell DNA synthesome, we probed the MDA MB-468 derived protein fractions with an antibody that

Table 3. DNA polymerase α and *in vitro* DNA replication activities of the DNA synthesome from nude mouse tumor tissue.

FRACTION	NE/S-3	S-4	P-4
DNA polymerase α[a]	40.6	2.0	123.2
DNA Replication +T[b]	57.2	11.2	158.7
DNA Replication –T[b]	5.5	4.1	9.6

[a]DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures. One unit of DNA polymerase activity is equivalent to $1x10^{-10}$ mol of ^{3}[H]-TMP incorporated into DNA per hour at 35°C. These values represent the average of two independent experiments.

[b]*In vitro* SV40 DNA replication assays were performed as described previously. One unit of replication activity equals the incorporation of 1 pmol of ^{32}[P]-dCMP into SV40 origin containing DNA. These values represent the average of two independent experiments.

recognizes the >200 kDa polypeptide. Immunoblot analysis reveals that DNA polymerase ε was present in the replication-competent P-4, Q-Sepharose peak and sucrose gradient peak fractions (Figure 7). Only a minor amount of DNA polymerase ε was present in the replication-deficient S-4 fraction (Figure 7), while none was detected in the Q-Sepharose flow-through (data not shown).

DNA Replication Fidelity of the Breast Cancer Cell DNA Synthesome.

The fidelity of DNA synthesis is mediated in part by the proof-reading capacity of the intrinsic 3'-5' exonuclease activity of DNA polymerase δ [25]. We employed a forward mutagenesis assay to measure the fidelity of the *in vitro* DNA synthesis process carried out by the breast cancer cell DNA synthesome (Materials and Methods) [35]. In this assay we utilized the DNA synthesome isolated from MDA MB-468 breast cancer cells and human breast tumor tissue to replicate plasmid DNA containing the SV40 origin of replication and the *lac-Zα* gene. The results of the fidelity assay were quantitated using the blue/white selection protocol described in the Materials and Methods [25]. These results were compared to the replication fidelity of the DNA synthesome isolated from nonmalignant Hs587Bst breast cells. We determined that the DNA synthesome purified from MDA MB-468 cells possessed a replication fidelity approximately 6 fold lower than that of the synthesome from Hs587Bst cells (Table 4). Similarly, the DNA synthesome purified from human breast tumor tissue possessed an approximately 5-fold lower DNA replication fidelity than the synthesome from Hs587Bst cells (Table 4). These differences in replication fidelity between the malignant and non-malignant breast cell DNA synthesome suggest that transformation to the malignant phenotype alters the process by which the synthesome from normal cells replicates DNA.

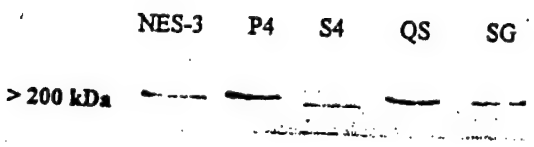


Figure 7. Immunoblot analysis for the presence of DNA polymerase ε in the MDA MB-468 breast cancer cell derived fractions. 50 μg of each protein fraction (PEG NE/S-3, S-4, P-4, Q-Sepharose peak (QS) and sucrose gradient peak (SG)) was resolved on a 8% polyacrylamide gel then transferred to a nitrocellulose membrane. The membrane was incubated with a primary antibody against human DNA polymerase ε. Following incubation with an anti-mouse secondary antibody conjugated to horseradish peroxidase, the immobilized protein was detected using a light-enhanced chemiluminescent system (Amersham).

Table 4. DNA replication fidelity of the breast cell DNA synthesome.

Origin of DNA Synthesome	% Mutants (average per 10^4 colonies)
MDA MB 468 breast cancer cell line	1.20% +/- 0.2%
Human breast tumor tissue	0.93% +/- 0.3%
Hs578Bst non-malignant breast cell line	0.19% +/- 0.08%

An *in vitro* DNA replication fidelity assay [25] was performed to measure the fidelity with which the DNA synthesome from MDA MB 468 breast cancer cells, human breast tumor tissue and Hs578Bst cells replicates plasmid DNA. The replicated plasmid, containing the bacterial lac-Z gene, was *DpnI* digested and electroporated into E.coli. The bacteria were then plated onto LB agar containing the chromogenic substrate X-gal. Transformed bacteria expressing a non-mutated lac-Z gene (encodes the B-galactosidase enzyme) formed blue colonies on the plate, while bacteria containing DNA with mutations in the lac-Z gene formed white colonies. Mutations occurring within the plasmid at locations other than the lac-Z gene are not detected by this assay. Consequently, the reported percentages of white colonies provide a minimum estimate of the actual number of mutations arising during DNA synthesome mediated DNA replication. The percentage of mutant colonies expressed for the DNA synthesome is the average number taken from 3 separate assays of 10^4 transformed colonies each. The background mutation frequency for the forward mutagenesis assay was determined to be 0.0003% (Materials and Methods).

Discussion

In this report, we have described for the first time the purification of a multiprotein DNA replication complex isolated from human breast cancer cells and breast tumor tissues. The integrity of the breast cancer cell DNA synthesome was maintained after its treatment with high salt, polyethylene glycol precipitation, anion-exchange chromatography and sucrose gradient sedimentation. These results suggest that the co-purification of the synthesome's proteins with one another is independent of non-specific interactions with other cellular macromolecules. In addition, upon velocity sedimentation analysis of the breast cancer cell DNA synthesome, both the DNA polymerase α and DNA replication activities co-migrated in the sucrose gradient with a coefficient of 18S. This 18S sedimentation coefficient is comparable to that obtained for the HeLa cell DNA synthesome [13,14].

Our data show that the DNA polymerase α and DNA replication activities of the synthesome isolated from breast cancer cells and breast tumor tissues were enriched by the successive steps of the purification process. Furthermore, the P-4 and Q-Sepharose peak fractions from breast tumor tissues possessed levels of *in vitro* SV40 DNA replication activity comparable to those found in fractions derived from breast cancer cells. Overall, the isolation of the DNA synthesome as a fully functional complex from human breast tumor tissues strongly suggests that the synthesome mediates DNA replication *in vivo*.

We have identified several of the key DNA replication proteins comprising the breast cancer cell DNA synthesome utilizing immunoblot analyses and enzymatic assays; these proteins include: DNA polymerase δ, PCNA, DNA polymerase α, DNA primase,

RF-C, RP-A, DNA topoisomerases I, II, and DNA polymerase ε. All of these polypeptides, excluding DNA polymerase ε, have been shown to be required for the faithful replication of SV40 DNA *in vitro* [8-10]. Moreover, the functions that each of these proteins performs during DNA replication have been determined by utilizing the SV40 system. Recent studies demonstrate that DNA polymerase α-primase synthesizes RNA-DNA primers required for the initiation of leading strand and Okazaki fragment synthesis [36,37]. On the other hand, DNA polymerase δ conducts the replication of the leading strand and completes synthesis of the lagging strand during DNA chain elongation [36,38]. According to a current model for eukaryotic DNA replication, the activities of both DNA polymerases α and δ are coordinated in part by RF-C, which serves as a connector or hinge between the proteins [27]. Additionally, PCNA, an accessory factor for polymerase δ, may participate in the coordination of leading and lagging strand synthesis by functioning as part of a molecular switch from the initiation to the elongation phase of DNA replication [38,39]. The co-purification of DNA polymerases α, δ, DNA primase, PCNA and RF-C with the breast cancer cell DNA synthesome indicates that the synthesome may act as a coordinated dipolymerase replication complex.

RP-A functions during SV40 DNA synthesis to stabilize newly formed single-stranded regions created in replicating DNA by the helicase activity of the large T-antigen [40]. Topoisomerase I, also a component of the breast cancer cell DNA synthesome, relaxes positive DNA supercoils as they accumulate ahead of the replication fork [41]. Such an action is necessary for translocation of the replication machinery along template DNA during DNA synthesis. In addition to topoisomerase I, topoisomerase II

can carry out the unwinding activity required for the progression of the replication fork during SV40 DNA synthesis [41]. Furthermore, studies in which intact cells were incubated with topoisomerase II inhibitors demonstrate that the topoisomerase II is necessary for the decatenation of newly replicated daughter DNA molecules following DNA synthesis [42]. The enzyme most likely functions in these roles as a component of the DNA synthesome. Although not yet identified as components of the breast cancer cell DNA synthesome, DNA helicase and DNA ligase I were found to copurify with the synthesome isolated from HeLa and FM3A cells [14,15]. Both of these enzymes have been shown to be required for eukaryotic DNA replication [43,44]. We are presently characterizing the breast tumor tissue-derived synthesome with respect to its protein components. Presumably, all of the proteins comprising the breast cancer cell DNA synthesome copurify with the tumor tissue-derived synthesome, as it is fully capable of supporting SV40 DNA replication *in vitro.*

In order to preserve the integrity of the information contained in DNA, normal mammalian cells must replicate their DNA with an error frequency as low as 10^{-10} [33]. Such a high fidelity for DNA replication must be maintained by the DNA synthesis and DNA repair systems functioning within the cell. We utilized a forward mutagenesis assay [25] to examine the fidelity with which the breast cancer cell and human breast tumor tissue derived DNA synthesome replicates plasmid DNA containing the *lac-Zα* gene. This assay detects point mutations occurring within the *lac-Zα* gene as well as frame-shift mutations occurring in other positions on the plasmid. We found a 5-6 fold decrease in the replication fidelities of the DNA synthesome isolated from malignant breast cells and tissue compared to that of the DNA synthesome isolated from a non-malignant breast

cell line (Table 4). Our results are consistent with the observation that mammary cancer cells accumulate extensive genetic damage [45,46]. The significant difference in the replication fidelities between the malignant and nonmalignant breast cell DNA synthesome suggests that transformation alters the process by which the latter replicates and/or participates in the repair of DNA. Indeed, it has been demonstrated that specific DNA replication proteins are targets for molecular modification during cellular transformation [4]. For example, DNA polymerases α and ε purified from Novikoff hepatoma cells have altered physicochemical and catalytic properties compared to the respective polymerases isolated from normal liver cells [4]. During DNA synthesis, these altered molecular and catalytic properties may contribute to a decreased specificity for nucleotide selection by the polymerases, which in turn leads to an increased mutation rate. Importantly, we have determined by two-dimensional polyacrylamide gel electrophoresis that significant physical differences exist between the protein components of the DNA synthesome purified from malignant and nonmalignant breast cells (Bechtel et al., unpublished data.) We are currently conducting experiments to determine the precise molecular changes that occur to the components of the breast cell DNA synthesome during transformation. We fully expect these studies to advance our understanding of how DNA replication fidelity is reduced in breast cancer cells.

We previously described a model for the organization of the proteins comprising the DNA synthesome isolated from mouse mammary carcinoma (FM3A) cells and HeLa cells [14,15]. We can now extend this model to include the breast cancer cell DNA synthesome, based on the fractionation and column chromatographic profiles of its protein components (Figure 8). As DNA polymerases α, δ, ε, DNA primase and RF-C

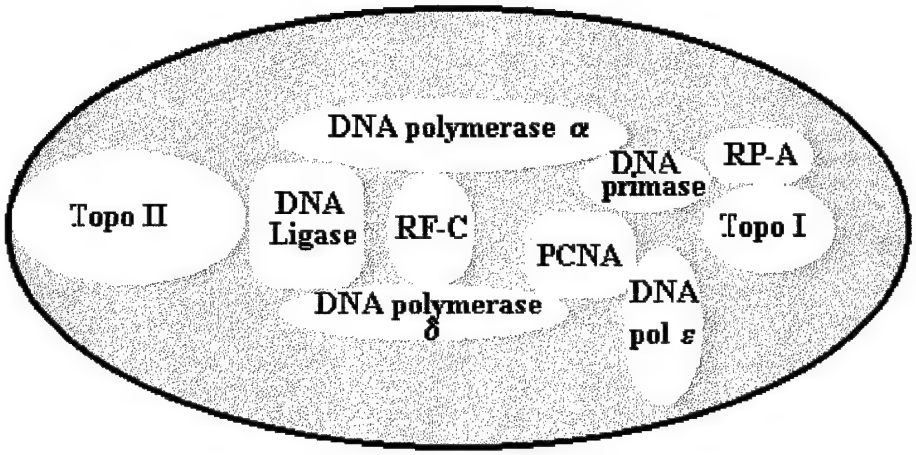


Figure 8. Model for the human breast cell DNA synthesome. A full description of the model is presented in the text.

were observed to copurify primarily with the replication-competent DNA synthesome, we propose that these proteins form the core of the DNA synthesome. The "tight" association of DNA polymerase ε with the DNA synthesome suggests that the protein may play a role in mammalian cell DNA replication. It has been postulated that DNA polymerase ε links the replication machinery with the S-phase checkpoint by acting as a sensor that coordinates transcriptional responses to DNA damage in yeast [47]. Such a role for the protein may exist in mammalian cells as well. In addition, we have included DNA ligase I as a member of the tightly associated components of the complex as it was observed to copurify exclusively with the DNA synthesome from FM3A and HeLa cells [14,15].

Unlike the other components, PCNA, RP-A and topoisomerases I and II were observed to co-fractionate and co-elute, following column chromatography, with those fractions containing the breast cancer cell DNA synthesome as well as with fractions lacking DNA replication activity. These results suggest that only a fraction of the cellular pools of PCNA, RP-A, and topoisomerases I, II, copurify with the DNA synthesome. This is consistent with the recognition that these proteins have additional roles in mediating cellular functions such as transcription, recombination and repair. During the initial stages of SV40 DNA replication, both topoisomerase I and RP-A facilitate the melting of SV40 DNA [48]. Therefore, we propose that both of these proteins constitute the "initiation" components of the breast cancer cell DNA synthesome. We are currently performing co-immunoprecipitation studies to determine the exact physical interactions of the synthesome's proteins with each other. Their physical association depicted in

Figure 8 is consistent with data from our laboratory as well as with several reports on SV40 and eukaryotic DNA replication [7,8,27,38].

In this report, we have isolated and described a multiprotein complex for DNA replication from breast cancer cells and breast tumor tissues. The isolation of a fully functional DNA synthesome from tumor tissues strongly suggests that the synthesome mediates breast cancer cell DNA replication *in vivo*. Furthermore, we have established that the human breast cancer cell and tumor tissue-derived DNA synthesome possess a lower fidelity for DNA replication than the synthesome purified from non-malignant breast cells. Breast cancer is characterized by a high proliferation phenotype and the accumulation of an extensive level of DNA damage within tumor cells [2,45,46]. Understanding the process of DNA replication, as it occurs in breast cancer cells, will greatly facilitate the development of improved anti-breast cancer therapies. We fully expect that the complete characterization of the breast cancer cell DNA synthesome will further our understanding of aberrant breast cell DNA replication as well as contribute to the development of these improved therapies.

Acknowledgements:

We are grateful to Yuetong Wei and QiQi Zhou for their technical expertise.

References

1. American Cancer Society, Basic Data, 1996.

2. Keshgegian, A. and Cnaan, A. Proliferation markers in breast carcinoma: mitotic figure count, S-phase fraction, proliferating cell nuclear antigen, Ki-67 and MIB-1. Anat Path 104:42-49; 1995.

3. Loeb, L. Mutator phenotype may be required for multistage carcinogenesis. Cancer Res. 51:3075-3079; 1991.

4. Popanda, O.; Fox, G.; Thielmann, H.W. DNA polymerases α, δ and ε of Novikoff hepatoma cells differ from those of normal rat liver in physicochemical and catalytic properties. J. Mol. Med. 73:259-268; 1995.

5. Kelly, T.J. SV40 DNA replication. JBC 263:17889-17892; 1988.

6. Challberg, M. and Kelly, T. Animal virus DNA replication. Annu Rev Biochem 58:671-717; 1989.

7. Stillman, B. Initiation of eukaryotic DNA replication *in vitro*. Annu Rev Cell Bio 5:107-245; 1989.

8. Hurwitz, J.; Dean, F.B.; Kwong, A.D.; Lee, S.H. The *in vitro* replication of DNA containing the SV40 origin. JBC 265:18043-18046; 1990.

9. Malkas, L.H.; Hickey, R.J.; Baril, E.F. Multienzyme complexes for DNA synthesis in eukaryotes: P-4 revisited. In: Strauss, P.R. and Wilson, S.H. (eds.) "The Eukaryotic Nucleus: Molecular Biochemistry and Macromolecular Assemblies." New Jersey, The Telford Press; 1990a: pp. 45-68.

10. Stillman, B.; Bell, S.P.; Dutta, A.; Marahrens, Y. In "Regulation of the Eukaryotic Cell Cycle" (Ciba Foundation Symposium 170) Chichester: John Wiley and Sons; 1992: pp. 147-156.

11. Matthews, C.K. and Slabaugh, M.B. Eukaryotic DNA metabolism: Are deoxynucleotides channeled to replication sites? Exp Cell Res 162:285-295; 1986.

12. Reddy, G. and Fager, R. Replitase: A complex integrating dNTP synthesis and DNA replication. Crit Rev Euk Gene Exp 3:255-277; 1993.

13. Malkas, L.H.; Hickey, R.J.; Li, C.J.; Pedersen, N.; Baril, E.F. A 21S enzyme complex from HeLa cells that functions in simian virus 40 (SV40) DNA replication *in vitro*. Biochem. 29: 6362-6374; 1990b.

14. Applegren, N.; Hickey, R.J.; Kleinschmidt, A.M.; Zhou, Q.; Coll, J.; Wills, P.; Swaby, R.; Wei, Y.; Quan, J.Y.; Lee, M.; Malkas, L.H. Further characterization of the human cell multiprotein DNA replication complex (MRC). J.Cell.Biochem. 59: 91-107; 1995.

15. Wu, Y.; Hickey, R.J.; Lawlor, K.; Wills. P.; Yu, F.; Ozer, H.; Starr, R.; Quan, J.Y.; Lee, M.; Malkas, L.H. A 17S multiprotein form of murine cell DNA polymerase mediates polyomavirus DNA replication *in vitro*. J.Cell. Biochem. 54: 32-46; 1994.

16. Tom, T.; Hickey, R.J.; Malkas, LH. Identification of multiprotein complexes containing DNA replication factors by native immunoblotting of HeLa cell protein preparations with T-antigen dependent SV40 DNA replication activity. J Cell Biochem (in press, 1996).

17. Yue, W.; Zhou, D.; Chen, S.; Brodie, A. A nude mouse model for postmenopausal breast cancer using MCF-7 cells transfected with the human aromatase gene. Cancer Res 54:5092-5095; 1994.

18. Simanis, V. and Lane, D.P. An immunoaffinity purification procedure for SV40 large T-antigen. Virology 144: 88-100; 1985.

19. Stillman, B.; Gerard, R.D.; Guggenheimer, R.A.; Gluzman, Y. T-antigen and template requirements for SV40 DNA replication *in vitro*. EMBO J. 4: 2933-2939; 1985.

20. Sambrook, J.; Fritsch, E.; Maniatis, T. In: Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; 1989.

21. LaMothe, P.; Baril, B.; Chi, A.; Lee, L.; Baril B. Accessory proteins for DNA polymerase α activity with single-strand DNA templates. PNAS 78:4723-4727; 1981.

22. Vishwanatha J.K.; Yamaguchi, M.; DePamphilis, M.L.; Baril, E.F. Selection of template initiation sites and the lengths of RNA primers synthesized by DNA primase are strongly affected by its organization in a multiprotein DNA polymerase α complex. Nuc Acid Res 14:7305-7323; 1986.

23. Hickey, R.J.; Malkas, L.H.; Pedersen, N.; Li, C.; Baril, E.F. Multienzyme complex for DNA replication in HeLa cells. Moses, R. and Summers, W. (eds.) In: DNA Replication and Mutagenesis, Amer. Soc. Microbiol. Public., Washington D.C.; 1988: pp. 41-54.

24. Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685; 1970.

25. Roberts, J.D. and Kunkel, T.A. The fidelity of a human cell DNA replication complex. PNAS 85:7064-7068; 1988.

26. Prelich, G.; Tan, C.K.; Mikostura, M.; Matthews, B.; So, A.G.; Downey, K.M.; Stillman, B. Functional identity of proliferating cell nuclear antigen and a DNA polymerase δ auxiliary protein. Nature (London) 326:517-520; 1987.

27. Tsurimoto, T. and Stillman, B. Replication factors required for SV40 DNA replication *in vitro*. JBC 266:1961-1968; 1991.

28. Lee, MYWT; Tan C.K.; Downey, K.M.; So, A.G. Further studies on calf thymus DNA polymerase δ purified to homogeneity by a new procedure. Biochem 23:1906-1913; 1984.

29. Lee S.H.; Kwong, A.D.; Pan, Z.Q.; Hurwitz, J. Studies on the activator-1 protein complex, an accessory factor for proliferating cell nuclear antigen-dependent DNA polymerase delta. JBC 266:594-602; 1991.

30. Henricksen, L.A.; Umbricht, C.B.; Wold, M.S. Recombinant replication protein A: Expression, complex formation and functional characterization. J. Biol. Chem. 269:11121-11132; 1994.

31. Fairman, M.P. and Stillman, B. Cellular factors required for multiple stages of SV40 DNA replication *in vitro*. EMBO J 7:1211-1218; 1988.

32. Hsiang, Y.H. and Liu, L.F. Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. Cancer Res. 48: 1722-1726; 1988.

33. Tuusa, J.; Uitto, L.; Syvaoja, J. Human DNA polymerase ε is expressed during cell proliferation in a manner characteristic of replicative DNA polymerases. Nuc Acid Res 23: 2178-2183; 1995.

34. Morrison, A.; Araki, H.; Clark, A.B.; Hamatake, R.B.; Sugino, A. A third essential DNA polymerase in S. *cerevisiae*. Cell 62: 1143-1151; 1990.

35. Roberts, J.D.; Thomas, D.C.; Kunkel, T.A. Exonucleolytic proofreading of leading and lagging strand DNA replication errors. PNAS 88:3465-3469; 1991.

36. Stillman, B. Smart machines at the DNA replication fork. Cell 78: 725-728; 1994.

37. Denis, D. and Bullock, P. Primer-DNA formation during simian virus 40 DNA replication *in vitro*. Mol. and Cell Bio. 13:2882-2890; 1993.

38. Waga, S. and Stillman, B. Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication *in vitro*. Nature 369:207-212; 1994.

39. Prelich, G. and Stillman, B. Coordinated leading and lagging strand synthesis during SV40 DNA replication *in vitro* requires PCNA. Cell 53:117-126; 1988.

40. Wobbe, C.R.; Dean, F.; Weissbach, L.; Hurwitz, J. *In vitro* replication of duplex circular DNA containing the simian virus 40 DNA origin site. PNAS 82:5710-5714; 1985.

41. Yang, L.; Wold, M.S.; Li, J.J.; Kelly, T.J.; Liu, L.F. Roles of DNA topoisomerases in simian virus 40 DNA replication. PNAS 84:940-954; 1987.

42. Snapka, R.M. Topoisomerase inhibitors can selectively interfere with different stages of simian virus 40 DNA replication. Mol. Cell. Bio. 6:4221-4227; 1986.

43. Budd, M.E.; Choe, W.C.; Campbell, A.L. DNA2 encodes a DNA helicase essential for replication of eukaryotic chromosomes. JBC 270:26766-9; 1995.

44. Petrini, J.H.; Xiao, Y.; Weaver, D.T. DNA ligase I mediates essential functions in mammalian cells. Mol. Cell. Bio. 15:4303-4308; 1995.

45. Thorgeirsson, S.S. Endogenous DNA damage and breast cancer. Cancer 71:2897-2899; 1993.

46. Nutter, L.M.; Wu, Y.Y.; Ngo, E.O.; Sierra, E.E.; Gutierrez, P.L.; Abul-Hajj, Y.J. An o-quinone form of estrogen produces free radicals in human breast cancer cells: correlation with DNA damage. Chem. Res. Tox. 7:23-28; 1994.

47. Navas, T.A.; Zhou, Z.; Elledge, S.J. DNA polymerase ε links the DNA replication machinery to the S phase checkpoint. Cell 80:29-39; 1995.

48. Boroweic, J.A.; Dean, F.B.; Bullock, P.A.; Hurwitz, J. Binding and unwinding: How T-antigen engages the SV40 origin of DNA replication. Cell 60:181-184; 1990.

Chapter 3

Citation: Oncology Res. 9: 629-639; 1997

MAPPING SPECIFIC PROTEIN-PROTEIN INTERACTIONS WITHIN THE CORE COMPONENT OF THE BREAST CELL DNA SYNTHESOME*

Jennifer M. Coll[a], Robert J. Hickey[a-d], Erica A. Cronkey[a], Hai-Yan Jiang[a], Lauren Schnaper[e,f], Marietta Y.W.T. Lee[g], Lahja Uitto[h], Juhani E. Syvaoja[h] and Linda H. Malkas[a-d+]

[a]Department of Pharmacology and Experimental Therapeutics, [b]Program in Molecular and Cellular Biology, [c]Program in Oncology, [d]Program in Toxicology, [e]Department of Surgery, University of Maryland at Baltimore, School of Medicine, Baltimore, MD 21201; [f]Breast Evaluation and Treatment Center, Greater Baltimore Medical Center, Baltimore, MD 21240; [g]New York Medical College, Department of Biochemistry and Molecular Biology, Valhalla, NY 10595; [h]Biocenter Oulu and Department of Biochemistry, University of Oulu, FIN 90570 Oulu, Finland.

Running Title: Breast Cancer Cell Multiprotein Complex for DNA Replication

Key Words: breast cancer; DNA replication; multiprotein complex; DNA polymerase; RF-C; PCNA; Poly(ADP-ribose) polymerase (PARP); RP-A; DNA synthesome; co-immunoprecipitation

ABSTRACT

We have previously described the isolation and characterization of an intact multiprotein complex for DNA replication, designated the DNA synthesome, from human breast cancer cells and biopsied human breast tumor tissue (Coll et al., 1996, Oncology Research **8**:435-447). The purified DNA synthesome was observed to support full DNA replication *in vitro*. We had also proposed a model for the breast cell DNA synthesome, in which DNA polymerases α, δ and ε, DNA primase and replication factor C (RF-C) represent members of the core component, or tightly associated proteins of the complex. This model was based on the observed fractionation, chromatographic and sedimentation profiles for these proteins. We report here that poly(ADP-ribose) polymerase (PARP) and DNA ligase I are also members of the breast cell DNA synthesome core component. More importantly, in this report we present the results of co-immunoprecipitation studies that were designed to map the protein-protein interactions between several members of the core component of the DNA synthesome. Consistent with our proposed model for the breast cell DNA synthesome, our data indicate that DNA polymerases α and δ, DNA primase, RF-C, as well as proliferating cell nuclear antigen (PCNA) tightly associate with each other in the complex; whereas, DNA polymerase ε, PARP and several other components were found to interact with the synthesome via a direct contact with only PCNA or DNA polymerase α. The association of PARP with the synthesome core suggests that this protein may serve a regulatory function in the complex. Also, the co-immunoprecipitation studies suggest that the three DNA polymerases α, δ and ε all participate in the replication of breast cell DNA. To our knowledge this is the first report ever to describe the close physical association of

polypeptides constituting the intact human breast cell DNA replication apparatus.

Title Footnote:

* This work was supported in part by research awards CA57350, CA65754 and CA73060 from the National Institutes of Health/National Cancer Institute to LHM. Also a University of Maryland School of Medicine Designated Research Initiation Fund award to LHM and a University of Maryland Cancer Center Research Award to RJH supported this work. JMC is a recipient of a U.S. Army Breast Cancer Research Program Predoctoral Fellowship.

+ **Author: Linda H. Malkas,** author to whom correspondence should be addressed

Address: University of Maryland School of Medicine
(a) Department of Pharmacology and Experimental Therapeutics
(b) Program in Molecular and Cellular Biology
(c) Program in Oncology
(d) Program in Toxicology
685 W. Baltimore St.
Baltimore, MD 21201

Tel: (410) 706-2313 or 1798
Fax: (410) 706-0032

Author: Jennifer M. Coll

Address: University of Maryland at Baltimore
(a) Department of Pharmacology and Experimental Therapeutics

Author: Robert J. Hickey

Address: University of Maryland School of Medicine
(a) Department of Pharmacology and Experimental Therapeutics
(b) Program in Molecular and Cellular Biology
(c) Program in Oncology
(d) Program in Toxicology

Author: **Erica A. Cronkey**

Address: University of Maryland at Baltimore
(a) Departrnent of Pharmacology and Experimental Therapeutics

Author: **Hai-Yan Jiang**

Address: University of Maryland at Baltimore
(a) Department of Pharmacology and Experimental Therapeutics

Author: **Lauren Schnaper**

Address: Greater Baltimore Medical Center
(f) Breast Evaluation and Treatment Center
(e) Department of Surgery

Author: **Marietta Y.W.T. Lee**

Address: New York Medical College
(g) Department of Biochemistry and Molecular Biology

Author: **Lahja Uitto**

Address: University of Oulu
(h) Biocenter Oulu and Department of Biochemistry

Author: **Juhani E. Syvaoja**

Address: University of Oulu
(h) Biocenter Oulu and Department of Biochemistry

Abbreviations used: SV40, simian virus 40; PCNA, proliferating cell nuclear antigen; RP-A, replication protein A; RF-C, replication factor C; PARP, poly(ADP-ribose) polymerase; PBS, phosphate buffered saline.

INTRODUCTION

Breast cancer progression is associated with the occurrence of numerous genetic alterations that favor the uncontrolled proliferation of mammary cancer cells (1). Inactivating mutations in the *p53* or *cyclin D* genes, for example, abrogate cell cycle check points and lead to increased cellular proliferation rates and the accumulation of extensive DNA damage (2). Among the markers used in breast carcinoma, a high tumor proliferation rate correlates closely with poor prognosis for overall patient survival (3). Therefore, elucidating the molecular mechanisms of breast cell DNA replication could be critical to the development of improved anti-breast cancer therapies.

To date, several reports have described the isolation of large macromolecular complexes of replication-essential polypeptides from the extracts of eukaryotic cells (4-7). For example, a megacomplex of enzymes involved in dNTP synthesis and DNA polymerization, known as replitase, has been purified from Chinese hamster embryo fibroblast cells (CHEF/18) (5). In other work, Jackson and Cook (8) reported the retention of a large megacomplex containing DNA polymerase α and other enzymes for DNA synthesis in agarose entrapped nuclei. The megacomplex could function in the replication of endogenous chromosomal DNA, and some evidence was obtained suggesting that it associates with the nuclear matrix (9). Additionally, the isolation of 100-150S megacomplexes from regenerating rat liver that contain DNA polymerase α-primase and associate with the nuclear matrix has been reported (7). Our laboratory was the first to isolate and characterize a fully functional 18-21S multiprotein complex for DNA synthesis, designated the DNA synthesome, from human cervical cancer (HeLa) cells as well as from murine mammary carcinoma (FM3A) and human leukemia (HL60)

cells (10-13). We have also recently reported that the DNA synthesome can be isolated successfully from human breast cancer cells and breast tumor tissue (14). Importantly, in all cases the DNA synthesome was shown to fully support semi-conservative papovavirus DNA replication *in vitro,* in the presence of the viral large T-antigen and plasmid DNA containing papovavirus origin sequences. Because papovaviruses are extensively dependent on the host cell DNA replication apparatus for their own viral genome synthesis, this indicates that the DNA synthesome must also function in mammalian cell DNA replication.

We have also demonstrated that the integrity and function of this multiprotein complex is maintained after its treatment with high-salt, non-ionic detergents, RNase, DNase, anion exchange chromatography, sedimentation through glycerol or sucrose gradients and electrophoresis through native polyacrylamide gels (10-15). These results indicate that the components of the DNA synthesome associate with each other independent of non-specific interactions with other cellular macromolecules.

The coordinated activities of several proteins have been shown to be required for eukaryotic DNA synthesis *in vitro* [reviewed in (6,16,17)]. These proteins have been shown to co-purify with the DNA synthesome (10-14) and include: DNA polymerases α, δ and ε, PCNA, RF-C, RP-A, topoisomerases I and II, and DNA primase. A model representing the synthesome has been proposed based on the observed fractionation, chromatographic and sedimentation profiles of the proteins that co-purified with the complex (11-14). The synthesome's core component was proposed to include the replication elongation proteins DNA primase, RF-C and DNA polymerases α, δ and ε due to their exclusive partitioning with one another during the course of synthesome

purification. In this report we describe the first experimental evidence that indicates a close physical association between several protein members of the proposed breast cell synthesome core component. This is important since models for the organization of DNA synthetic proteins into replicating machines have been proposed (18); however, it is still unclear how these replication factors associate with one another to facilitate the efficient and coordinated process of DNA synthesis in mammalian cells. This report describes studies aimed at beginning to address this problem.

MATERIALS AND METHODS

Cell Culture. Suspension cultures of MDA MB-468 human breast cells were adapted from monolayer cultures. The cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of irradiated new-born calf serum and fetal bovine serum. Exponentially growing cells (5x 10^5 cells/ml medium) were harvested and washed three times with PBS (19): 20 mM Na_2HPO_4, 0.15 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4. The cells were then pelleted by low-speed centrifugation (1000rpm, 5 minutes, 4°C), and the cell pellets stored at -80°C until fractionation.

Isolation and Purification of the DNA synthesome from MDA MB-468 Breast Cancer Cells and Human Breast Tumor Tissue. The DNA synthesome was isolated from MDA MB-468 cells (20 g) and an infiltrating type ductal carcinoma of the female mammary gland according to our previously published procedures (14). We have shown previously that the NE/S-3, P4 and Q-Sepharose (Pharmacia, Piscataway, NJ) peak fractions contain the replication-competent DNA synthesome, whereas the S-4 and Q-Sepharose anion-exchange column flow-through fractions do not contain the complex.

***In vitro* SV40 DNA Replication Assay**. *In vitro* DNA replication assays were performed according to previously published procedures (20). One unit of SV40 replication activity is equivalent to the incorporation of 1 pmol dCMP into newly synthesized DNA per 2h under the standard assay conditions.

Purification of SV40 Large T-antigen. SV40 large T-antigen was purified from 293 cells infected with a recombinant adenovirus vector, Ad-SVR284, as detailed elsewhere (21).

Enzyme Assays. DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures (22,23). One unit of DNA polymerase α activity is equivalent to the incorporation of 1nmol [^{3}H]-TMP into DNA per hour at 37°C. **DNA primase** activity with single-stranded poly(dT) template DNA was also assayed according to previously published procedures (23). One unit of DNA primase activity is equal to the incorporation of 1 nmol [^{3}H]-dAMP into DNA per hour at 30°C.

Co-immunoprecipitation of the Core Protein Members of the DNA Synthesome by PCNA and DNA Polymerases α and δ Antibodies. Antibody sources: anti-RF-C, a generous gift from Dr. Bruce Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); anti-PARP, a generous gift from Dr. Mark Smulson (Georgetown University School of Medicine, Washington, D.C.); anti-RP-A, a generous gift from Dr. Bruce Stillman; and anti-DNA ligase I, a generous gift from Dr. Dana Lasko (Lady Davis Institute for Medical Research, Montreal, Quebec). One hundred micrograms of the Q-Sepharose peak fraction from MDA MB-468 breast cancer cells or 50 μg of the P-4 fraction from human breast tumor tissue was precleared with either protein A or G-conjugated agarose beads for 10 minutes at 4°C. The precleared protein fractions were next incubated overnight at 4°C with monoclonal antibodies directed against PCNA (mAb-10; 2.5 μg/reaction), DNA polymerase δ (mAb-11; 80 μl tissue culture supernatant/reaction) or DNA polymerase α (SJK 132-20 (24); 20 μg purified ascites/reaction). For negative controls, co-immunoprecipitations were also performed with a monoclonal antibody directed against the PDGF receptor (1 μg/reaction) and pre-immune mouse IgG (0.5 μg/reaction). Eighty microliters of a 10% suspension of protein

A or G conjugated-agarose beads, resuspended in buffer 1 [500mM NaCl, 25mM Tris-HCl (pH 7.5), 5mM EDTA (pH 7.5) and 1% Triton X-100], was then added to the respective reactions and the incubations continued for an additional 2 hours at 4°C. The reaction mixtures were then centrifuged at 3500 rpm for five minutes at 4°C, and the supernatant and pellet fractions were reserved. Subsequently, the pellet fraction was washed three times with buffer 1, then twice with a low-salt buffer [5mM EDTA (pH 7.5) and 10mM Tris-HCl (pH 7.5)]. Both the pellet and supernatant fractions were heat denatured for 1 minute at 100°C in Laemmli loading buffer, then subjected to SDS-PAGE (100 volts). After the electrophoretic transfer of the separated polypeptides to nitrocellulose membranes (20 volts, 16 hours, 4°C), the following antibodies were used to probe the blots: anti-PCNA (1:500; recognizes the 36 kDa protein); anti-DNA polymerase δ (1:3; recognizes the 125 kDa polypeptide); anti-DNA polymerase α (1:250; recognizes the 180 kDa polypeptide) (24); anti-DNA polymerase ε (1:500; recognizes the >200 kDa polypeptide); anti-RF-C (1:500; recognizes the 140 kDa polypeptide); anti-PARP (1:750; recognizes the 116 kDa polypeptide); anti-RP-A (1:500; recognizes the 34 kDa polypeptide); and anti-DNA ligase I (1:750; recognizes the 110 kDa polypeptide). Each blot was next incubated with the appropriate species-specific horseradish peroxidase conjugated secondary antibody and the immunodetection of the replication proteins performed with a light-enhanced chemiluminescence system. In the experiments where the Q-Sepharose peak supernatants were assayed for DNA polymerase α, DNA primase and *in vitro* DNA replication activities, co-immunoprecipitations were performed as follows. Briefly, 50 μg of the Q-Sepharose peak fraction was incubated with either 20 μg of PCNA antibody conjugated to protein A agarose beads, 20 μl of DNA polymerase

α antibody (purified ascites) or 5 μg of pre-immune mouse IgG for 4 hours at 4°C on an orbital shaker. Protein G agarose beads (30 μl), pre-coated with BSA, were next added to the reactions containing polymerase α antibody or pre-immune mouse IgG, and the incubations continued for an additional 1 hour at 4°C. The antigen-antibody complexes, bound to the protein A or G conjugated agarose beads, were pelleted by low-speed centrifugation at 3500 rpm for 5 minutes at 4°C. The protein supernatants were collected from each reaction and assayed immediately for DNA polymerase α, DNA primase and *in vitro* SV40 DNA replication activities. The positive controls for each reaction, which were also incubated for 5 hours at 4°C, contained 50 μl of the Q-Sepharose peak fraction diluted to the appropriate level with PBS buffer.

Native Gel Electrophoresis and Native Immunoblotting. Native gel electrophoresis and native immunoblot analyses were performed as described in previously published procedures from our laboratory [15].

RESULTS

PARP and DNA Ligase I Are Part of the Breast Cell Synthesome Core Component.

We isolated the DNA synthesome from MDA MB-468 human breast cancer cells and biopsied human breast tumor tissue according to the fractionation scheme described in our previously published procedures (14). The breast-cell-derived NE/S-3, P-4, S-4, Q-Sepharose peak and flow-through fractions (Materials and Methods) were subjected to Western blot analyses for the detection of PARP and DNA ligase I (Figures 1A and B, respectively). It was observed that both PARP and DNA ligase I partition exclusively with the NE/S-3, P4 and Q-Sepharose peak fractions (Figures 1A, B). These proteins were not detectable in the S-4 or Q-Sepharose flow-through fractions. We have previously shown that those fractions enriched for the DNA synthesome, namely, the NE/S-3, P-4 and Q-Sepharose peak, are fully competent to support large T-antigen dependent SV40 DNA replication *in vitro* (14). It was also found that those proteins that are members of the core component of the synthesome model partition exclusively with these fractions (11-14). Therefore, our data reported here suggest that both PARP and DNA ligase I are also members of the core component of the breast cell DNA synthesome.

The Breast Cell DNA Synthesome Is a Discrete High-Molecular-Weight Complex in Native Polyacrylamide Gels.

Recent work from our laboratory indicates that the synthesome derived from HeLa cells could be isolated from native polyacrylamide gels as a discrete high molecular weight complex (Tom et al., to be published elsewhere). It has been found that this discrete high molecular weight band is significantly enriched for as a function of

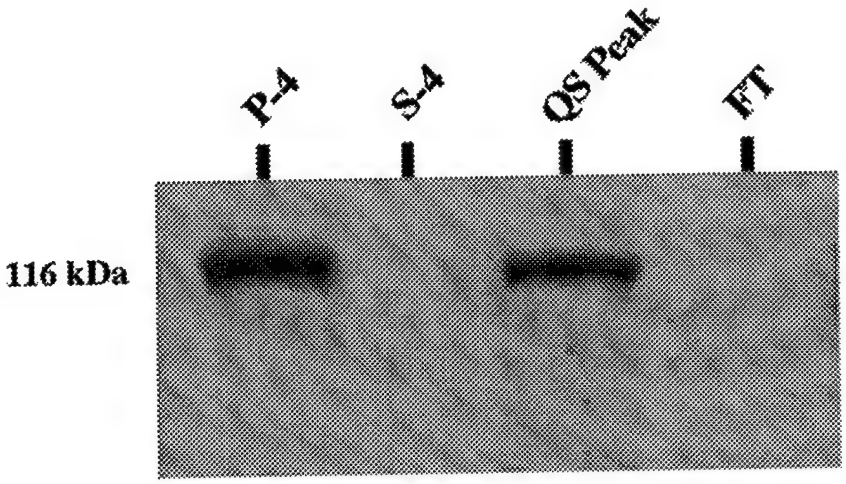


Figure 1A

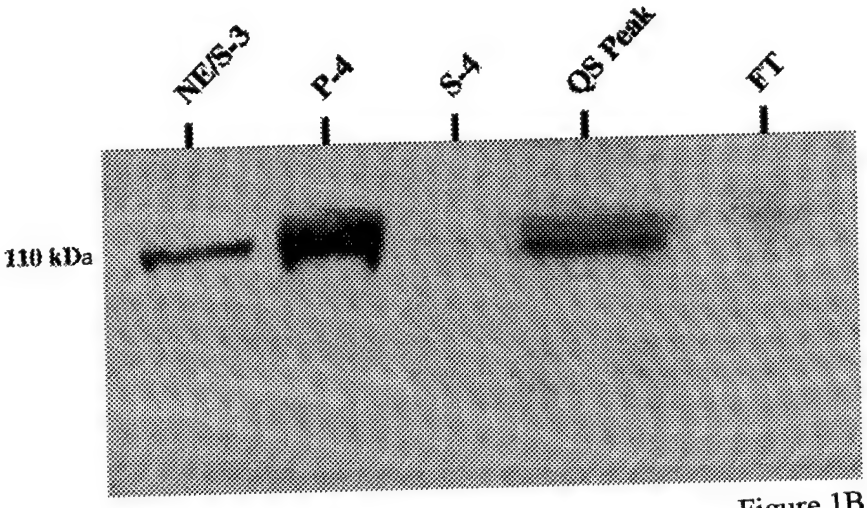


Figure 1B

Figure 1. Identification of **(A)** PARP and **(B)** DNA ligase I as core components of the breast cell DNA synthesome. Fifty micrograms of the NE/S-3, P-4, S-4, Q-Sepharose peak (QS peak) and Q-Sepharose flow-through (FT) protein fractions was resolved by SDS-PAGE (100 volts), then transferred to nitrocellulose membrane filters (20 volts, 16 hours, 4°C). Polyclonal antibodies directed against **(A)** PARP or **(B)** DNA ligase I were used at 1:750 dilutions to probe the membranes for either the 116- or 110-kDa proteins, respectively. Subsequently, each membrane was incubated with the appropriate species-specific horseradish peroxidase conjugated secondary antibody at a dilution of 1:5000. Immunodetection of the DNA replication proteins was performed using a light-enhanced chemiluminescence system (Amersham).

synthesome purification from HeLa cells. Also, this high molecular weight material has been electroeluted from native polyacrylamide gels and shown to contain several replication proteins (DNA polymerases α, δ, PCNA, topoisomerase I) and maintain its ability to support *in vitro* SV40 DNA replication. We report here, for the first time, that the synthesome from MDA MB-468 breast cancer cells (Figure 2, lanes 1 and 2) and human breast tumor tissue (data not shown), also migrates as a discrete high molecular weight band when resolved on native polyacrylamide gels.

Protein-Protein Interactions within the Breast Cell DNA Synthesome.

To test for close physical association between the protein members of the core component of the breast cell DNA synthesome, we performed co-immunoprecipitation studies (Materials and Methods) with the Q-Sepharose peak fraction using monoclonal antibodies directed against PCNA (mAb-10) and DNA polymerases α (SJK 132-20) and δ (mAb-11). PCNA serves as an accessory factor for DNA polymerases δ and ε, enhancing their enzymatic activities by forming a sliding clamp around duplex DNA (25,26). In accordance with its role as an auxiliary factor for DNA polymerases δ and ε, we found that PCNA antibody precipitated these two polymerases from the Q-Sepharose peak fraction in addition to the PCNA polypeptide (Figure 3, lanes 5 and 11). Also, DNA polymerase α and RF-C were co-precipitated by the PCNA antibody (Figure 3, lanes 7 and 9), evidence for a close interaction between PCNA and these proteins within the breast cell DNA synthesome. This result is supported by affinity chromatography studies performed with the bacteriophage T4 replication system in which a physical association between the T4 analogues of PCNA and RF-C was found (27). DNA ligase I, RP-A and PARP, on the other hand, were not co-precipitated by the PCNA antibody

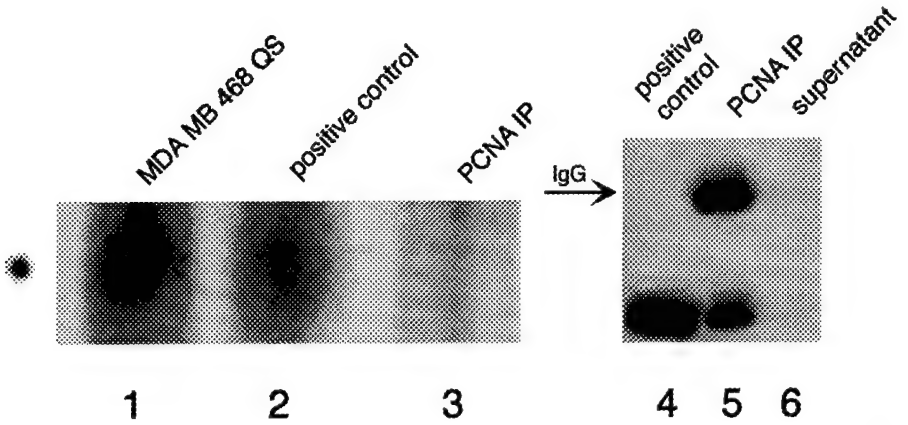


Figure 2

Figure 2. The breast cell DNA synthesome migrates as a discrete high molecular weight complex on native 4% polyacrylamide gels. Thirty micrograms of the Q-Sepharose peak (lane 1), the protein supernatant obtained after immunoprecipitation of PCNA polypeptide from 60 μl of the Q-Sepharose peak by PCNA antibody (mAb- 10; 2.5 μg) (lane 3), and a control for this reaction containing 60 μl of the Q-Sepharose peak diluted to the appropriate level with PBS buffer (lane 2), were resolved by native polyacrylamide gel electrophoresis (90 volts, 4°C). The resolved polypeptides were then transferred to nitrocellulose membrane filters (15 volts, 16 hours, 4°C). A monoclonal antibody directed against DNA polymerase α antibody (SJK 132-20) was used at a 1:400 dilution to probe the membrane for the high molecular weight band that represents the DNA synthesome. To demonstrate that PCNA antibody had completely precipitated PCNA polypeptide from the Q-Sepharose peak in these experiments, we subjected both the pellet (lane 5) and supernatant (lane 6) fractions obtained in a parallel immunoprecipitation experiment to SDS-PAGE. After electrophoretic transfer of the resolved polypeptides to nitrocellulose membrane filters, PCNA antibody was used at a 1:500 dilution to probe the membrane for the 36 kDa protein. Lane 4 shows PCNA contained in 50 μg of the Q-Sepharose peak (positive control).

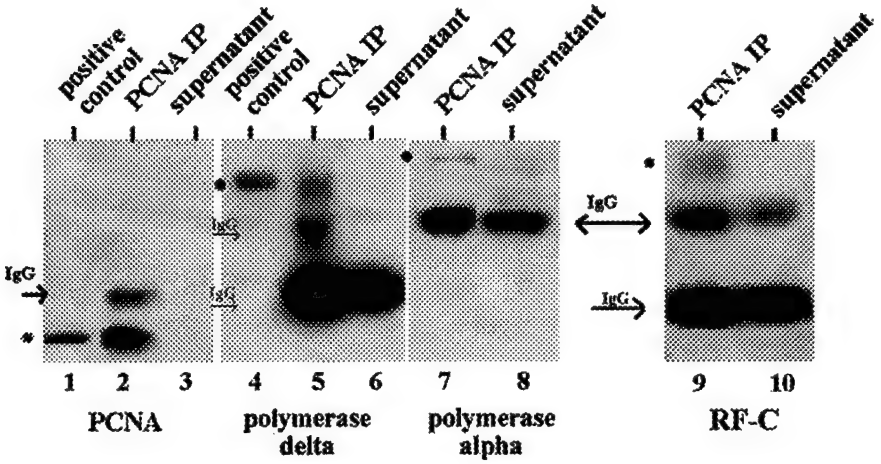


Figure 3, panel 1

Figure 3. Co-immunoprecipitation of several of the protein components of the DNA synthesome by a monoclonal antibody directed against PCNA (mAb-10). For each panel, the positive control lanes contained 50 μg of the Q-Sepharose peak probed with the respective antibodies. The target proteins are marked by a bullet and the antibody heavy and light chains (IgG) are marked by arrows. IP represents an abbreviation for immunoprecipitation. The bullets in lanes 22-24 mark PCNA, DNA polymerases α and δ, respectively.

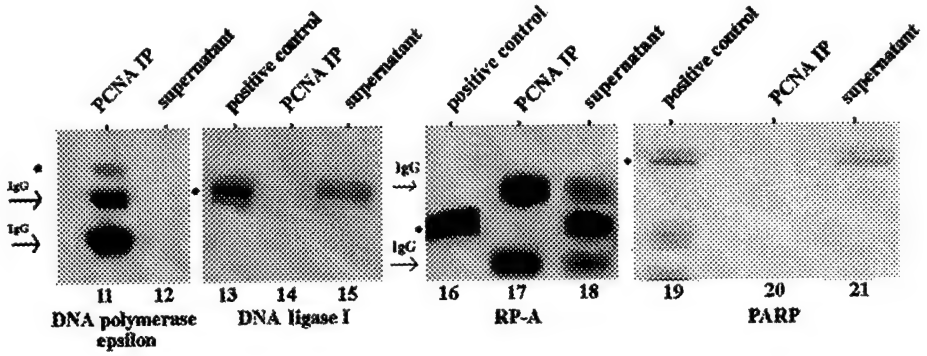


Figure 3, panel 2

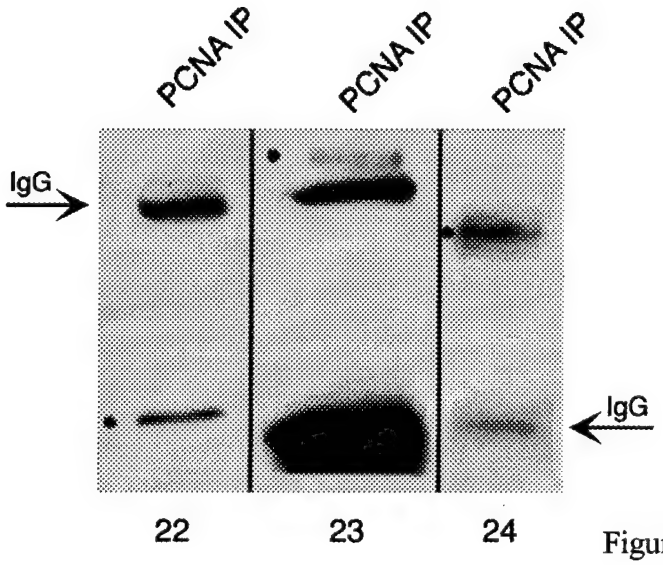


Figure 3, panel 3

(Figure 3, lanes 14, 17 and 20), suggesting that these polypeptides do not directly contact PCNA in the breast cell DNA synthesome.

Consistent with our results that PCNA interacts closely with several components of the DNA synthesome, PCNA antibody depleted the Q-Sepharose peak fraction of the discrete high-molecular-weight band that represents the DNA synthesome on native polyacrylamide gels (Figure 2, lane 3). Moreover, PCNA antibody precipitated PCNA as well as polymerases α and δ from the human breast tumor tissue derived DNA synthesome (Figure 3, lanes 22-24, respectively). These data from human breast tissue indicate that the protein interactions detected within the DNA synthesome from MDA MB-468 breast cancer cells do not merely represent a cell line dependent phenomenon. Further evidence for a close interaction between DNA polymerases α, δ, PCNA and RF-C within the breast cell DNA synthesome was obtained in immunoprecipitation experiments performed with DNA polymerase δ antibody (mAb-11). As shown in Figure 4, DNA polymerase δ antibody precipitated DNA polymerase δ from the Q-Sepharose peak fraction as well as PCNA, DNA polymerase α and RF-C proteins (Figure 4, lanes 1, 5, 7 and 9). Similar results demonstrating a close interaction between polymerases α, δ and RF-C within the RC complex from calf thymus were recently described (28). It has been proposed that synthesis of the leading and lagging strand DNA templates, mediated by DNA polymerases α and δ, respectively, is coordinated by RF-C, which acts as a molecular hinge between the two polymerases (29). Our experimental results are consistent with this theory. In contrast, we found that DNA polymerase ε, DNA ligase I, RP-A and PARP were not co-precipitated by the DNA polymerase δ antibody (Figure 4,

Figure 4, panel 1

Figure 4. Co-immunoprecipitation of several of the protein components of the DNA synthesome by a monoclonal antibody directed against DNA polymerase δ (mAb- 11). For each panel, the positive control lanes contained 50 μg of the Q-Sepharose peak probed with the respective antibodies. The target proteins are marked by a bullet and the antibody heavy and light chains (IgG) are marked by arrows. IP represents an abbreviation for immunoprecipitation.

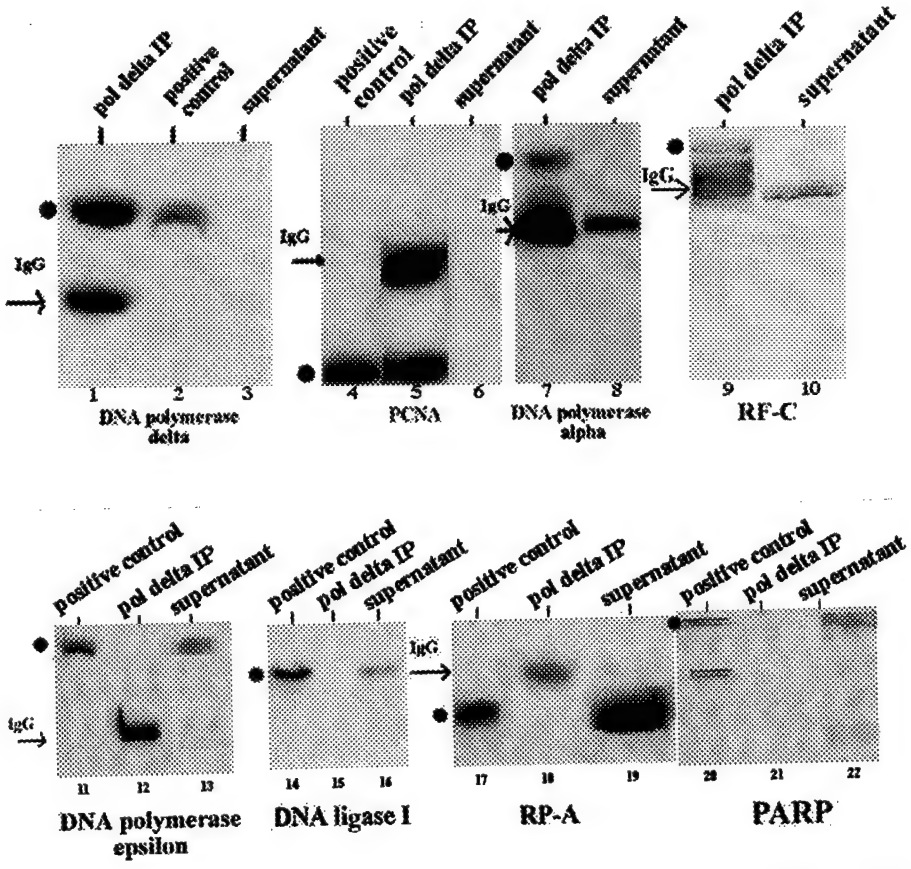


Figure 4, panel 2

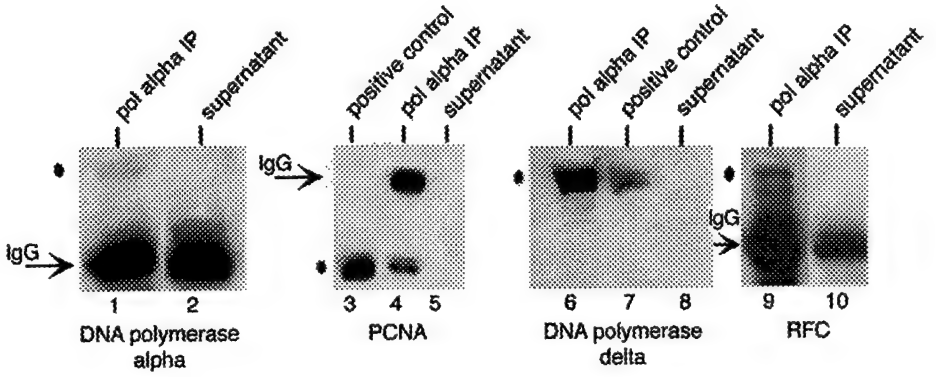
lanes 12, 15, 18 and 21) and probably do not directly contact DNA polymerase δ in the breast cell DNA synthesome.

As observed for the PCNA and DNA polymerase δ immunoprecipitations, DNA polymerases α and δ, PCNA and RF-C polypeptides were also precipitated by DNA polymerase α antibody (SJK 132-20) (Figure 5, lanes 1, 3, 7 and 9). Additionally, DNA polymerase α antibody co-precipitated RP-A, or single-stranded DNA binding protein (Figure 5, lane 18), and PARP (Figure 5, lane 21), which catalyzes the poly(ADP-ribosyl)ation of proteins involved in such nuclear processes as DNA replication and DNA repair (30). Direct evidence for a physical interaction between purified RP-A and DNA polymerase α-primase proteins has been reported by other laboratories (31). Furthermore, it has been shown that PARP and DNA polymerase α purify together in 400 and 700 kDa macromolecular complexes purified from calf thymus (32) as well as interact with each other in intact 3T3-L1 cells during the initial stages of their differentiation (33).

Several lines of evidence indicate that PARP may participate in the regulation of the initiation stage of DNA synthesis. First, PARP modulates the activities of DNA polymerase α (34), topoisomerase I (35) and RP-A (30), proteins that participate in the initiation events of DNA synthesis, by catalyzing their poly(ADP-ribosyl)ation. Second, PARP can inhibit the elongation of Okazaki fragments catalyzed by DNA polymerase α during SV40 DNA replication *in vitro* (30). We have recently reported that the overall *in vitro* replication activity of the DNA synthesome is modulated by PARP *per se* and/or poly(ADP-ribosyl)ation (36). We are currently conducting experiments to determine precisely how PARP modulates the activity of the breast cell DNA synthesome.

Figure 5, panel 1

Figure 5. Co-immunoprecipitation of several of the protein components of the DNA synthesome by a monoclonal antibody directed against DNA polymerase α (SJK 132-20). For each panel, the positive control lanes contained 50 μg of the Q-Sepharose peak probed with the respective antibodies. The target proteins are marked by a bullet and the antibody heavy and light chains (IgG) are marked by arrows. IP represents an abbreviation for immunoprecipitation.



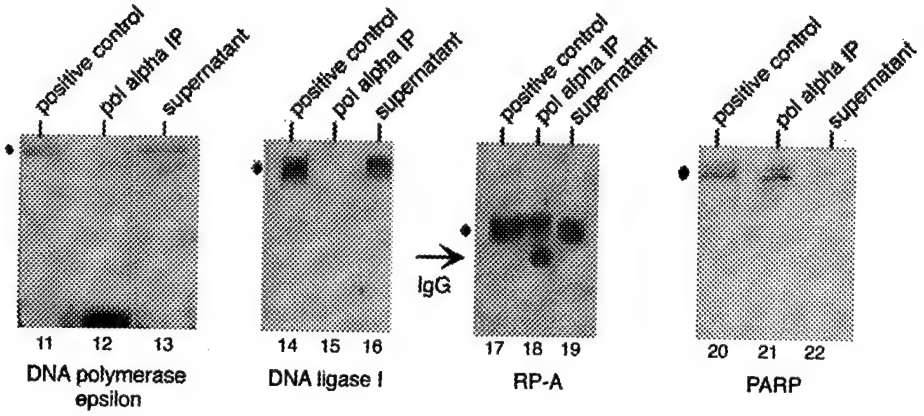


Figure 5, panel 2

Neither DNA polymerase ε nor DNA ligase I were co-precipitated by the DNA polymerase α antibody (Figure 5, lanes 12, 15). These results suggest that these proteins do not directly associate with DNA polymerase α in breast cell DNA synthesome. Most importantly, the protein associations detected by the immunoprecipitations with the PCNA, DNA polymerases δ and α antibodies were specific for these antibodies. None of the components of the breast cell DNA synthesome were precipitated by a monoclonal antibody directed against the platelet-derived growth factor (PDGF) receptor or pre-immune mouse IgG (Figure 6).

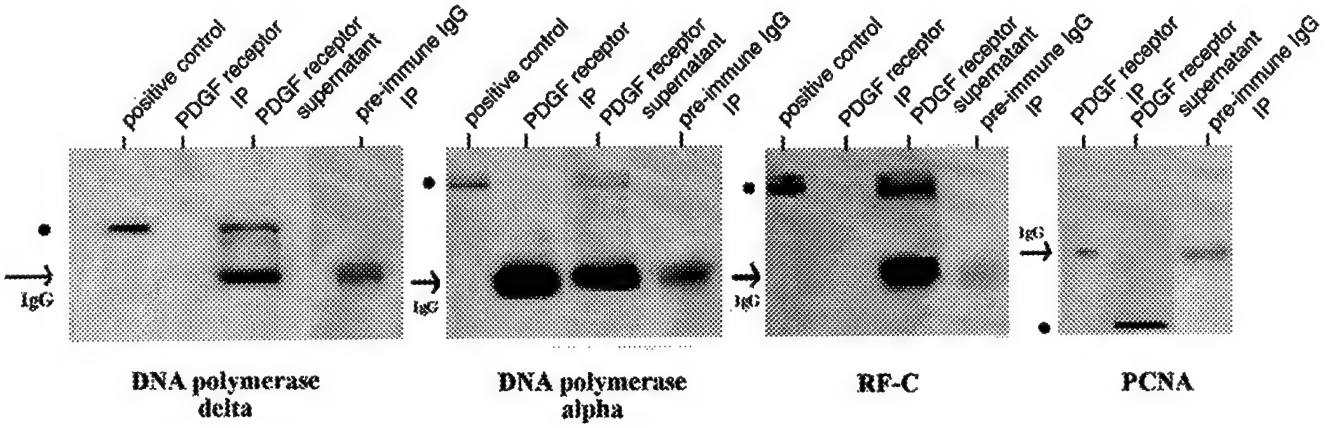
A summary of all of the co-immunoprecipitation results is presented in Table 1. From the data in this table several clear synthesome protein-protein interactions emerge.

DNA Polymerase α, DNA Primase and *in vitro* DNA Replication Activities of PCNA and DNA Polymerase α-Depleted Q-Sepharose Peak Fractions.

Finally, we also assayed PCNA and DNA polymerase α depleted Q-Sepharose peak fractions from MDA MB-468 breast cancer cells for DNA polymerase α, DNA primase and SV40 *in vitro* DNA replication activities. As shown in Figure 7 (lanes 2, 3, 5, 6, 8, 9), these fractions contained significantly reduced levels of DNA polymerase α, DNA primase and *in vitro* replication activities compared to control fractions. These data provide additional strong evidence for a physical association between PCNA and DNA polymerases α and δ within the breast cell DNA synthesome. Furthermore, these results indicate that DNA primase, the tightly associated subunit of DNA polymerase α, also associates with DNA polymerase δ and PCNA in the DNA synthesome. As a negative control, the Q-Sepharose peak was treated with mouse pre-immune IgG, which did not affect the ability of the breast cell DNA synthesome to support the respective enzymatic

Figure 6, panel 1

Figure 6. Negative control co-immunoprecipitations performed with a monoclonal antibody directed against the PDGF receptor or pre-immune mouse IgG. For each panel, the positive control lanes contained 50 μg of the Q-Sepharose peak probed with the respective antibodies. The target proteins are marked by a bullet and the antibody heavy and light chains (IgG) are marked by arrows. IP represents an abbreviation for immunoprecipitation.



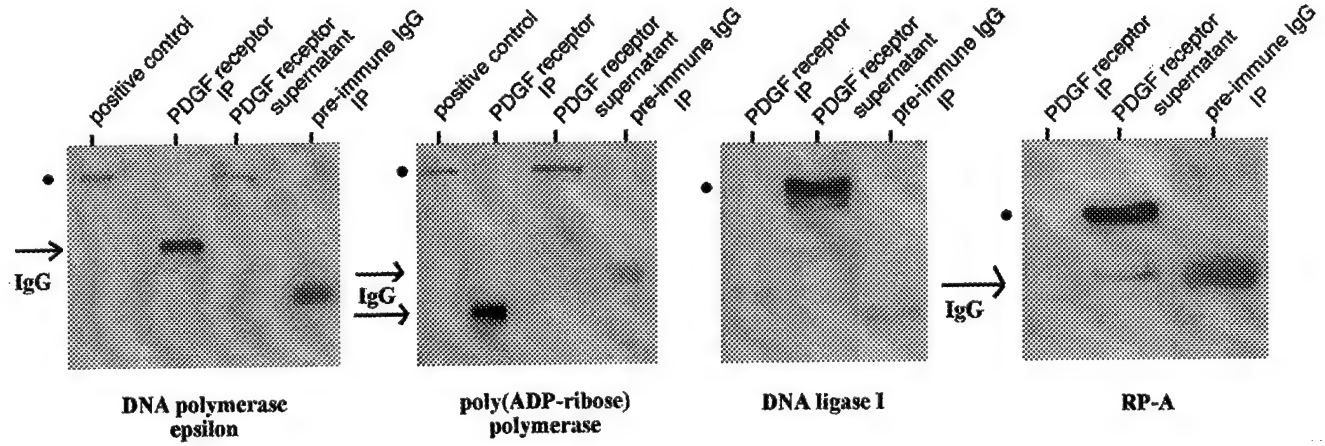
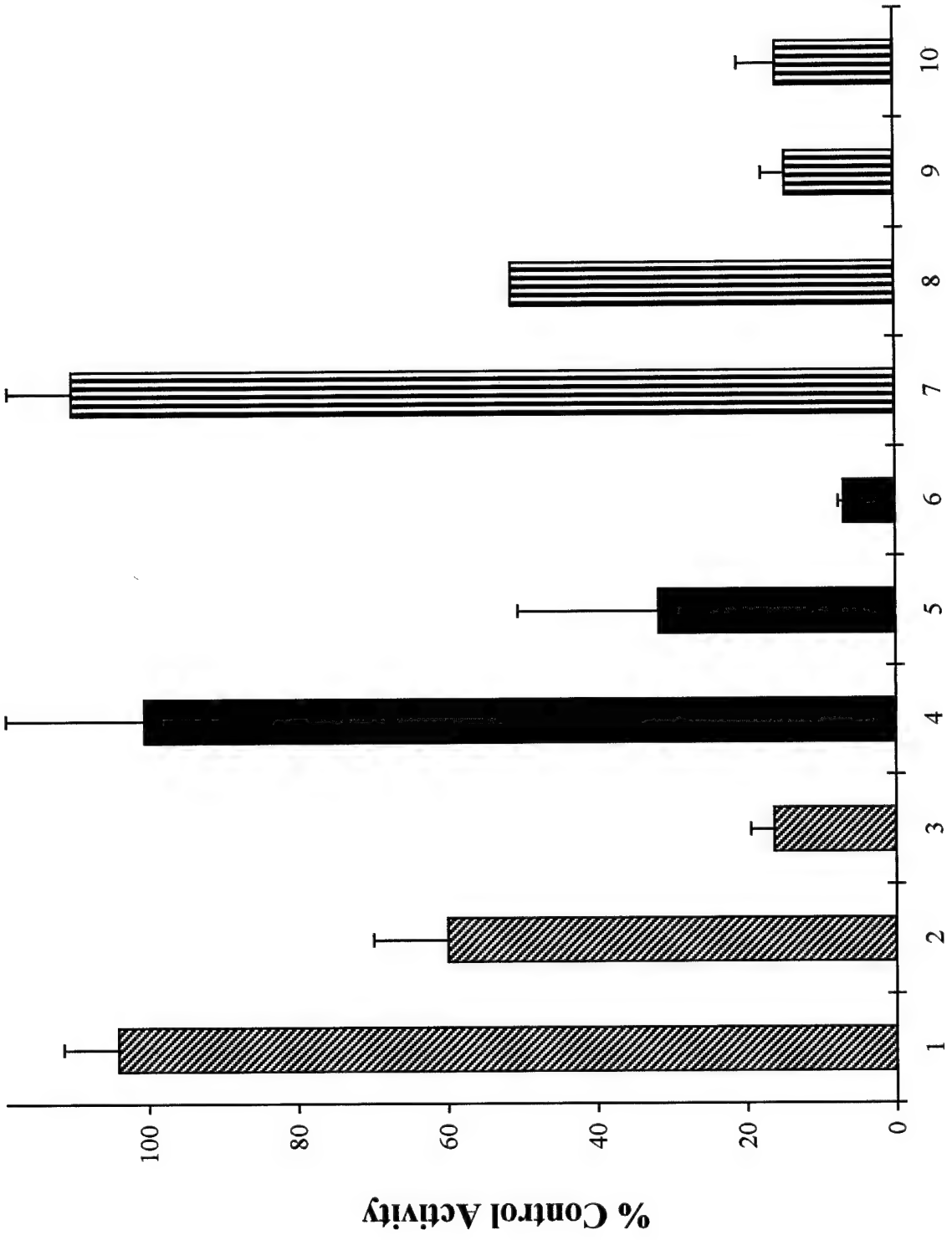


Figure 6, panel 2

Figure 7. DNA polymerase α, DNA primase and *in vitro* SV40 DNA replication activities of the Q-Sepharose peak fraction treated with monoclonal PCNA (mAb- 10) and DNA polymerase α (SJK 132-20) antibodies. The Q-Sepharose peak fraction was incubated with PCNA antibody conjugated to protein A agarose beads (lanes 2, 5 and 8), DNA polymerase α antibody (purified ascites) (lanes 3, 6 and 9) or pre-immune mouse IgG (lanes 1, 4 and 7). After the antigen-antibody-protein A/G agarose complexes were pelleted by low-speed centrifugation, the protein supernatants were collected and assayed immediately for DNA polymerase α, DNA primase and *in vitro* DNA replication activities. Diagonal striped bars represent DNA polymerase α activity. Solid bars represent DNA primase activity and vertical striped bars represent *in vitro* DNA replication activity. Each value for percent control activity (cpm) is an average of 2-5 separate experiments. Lines represent deviations from the average. Bar 10 represents the *in vitro* DNA replication activity contained in both PCNA and polymerase α depleted Q-Sepharose peak fractions. The typical control values for DNA polymerase α, DNA primase and *in vitro* DNA replication activities are 5,253 cpm, 8,083 cpm and 103,336 cpm, respectively.

Figure 7



and *in vitro* replication activities (Figure 7, lanes 1, 4, 7).

DISCUSSION

We have demonstrated that DNA polymerases α and δ, DNA primase, PCNA and RF-C tightly associate with each other in the breast cell DNA synthesome [as suggested in our previously proposed model of the complex (14)], as each of these proteins co-precipitates with antibodies directed against polymerases α, δ and PCNA. Our results are supported by the work of others previously reported for a DNA polymerization-competent, but not replication-competent, multiprotein complex purified from calf thymus (28) and by data obtained in reconstituted *in vitro* replication systems (29,37). However, this is the first report ever to describe the close physical association of DNA replication proteins within an intact replication-competent multiprotein complex isolated from human cancer cells and tumor tissue. When we began these studies it was initially anticipated that a single antibody directed against one of the synthesome replication proteins might coprecipitate all of the protein components of the complex. However, our experimental results have shown that this is not the case. Only those synthesome proteins that are in close physical association with a target protein are able to be coprecipitated under the conditions used in these studies. It may be somewhat naïve to assume that a single antibody would be able to "catch" the entire synthesome. It is possible that the binding of the antibody itself to one component of the synthesome could promote the disruption of some of the interactions between other components of the complex that are distal to the target protein bound by the antibody. Or perhaps the shape of the synthesome itself may cause a partial disassembly of the complex under the coimmunoprecipitation conditions. Experiments are currently underway in our laboratory to use two antibodies directed at different synthesome targets in an effort to "catch" the entire complex under

coprecipitation conditions. Nonetheless, the results reported here are important and offer the first detailed glimpse at how a significant component of the synthesome is composed.

We have previously proposed that DNA polymerases α, δ, ε, DNA primase and RF-C constitute the core of the breast cell DNA synthesome as these polypeptides co-purify with only those fractions enriched for the DNA synthesome during the purification process (14). Based on the similar fractionation profiles for PARP and DNA ligase I, we now include these proteins members of the core component of the breast cell DNA synthesome. Furthermore, PCNA may also represent a core component of the breast cancer cell synthesome as it was found to associate tightly with DNA polymerases α, δ, ε, DNA primase and RF-C. The presence of PCNA in those fractions not enriched for the DNA synthesome (11-14) may be interpreted to suggest that more than one pool of PCNA exists within the cell. In support of this claim, it has previously been reported for HeLa cells that only about 35% of the total cellular PCNA associates with replication foci during the peak of the S-phase (38).

We have constructed a new model for the organization of the proteins within the breast cell DNA synthesome based on the data presented in this report (Figure 8). Overall, our data suggest that the breast cell DNA synthesome represents an asymmetric multiprotein complex for DNA replication. According to one model for DNA replication *in vitro,* polymerase α-primase synthesizes RNA primers required for the initiation of leading strand and Okazaki fragment synthesis, whereas DNA polymerase δ conducts leading and lagging strand DNA synthesis during the elongation phase of DNA replication (37). It is postulated that RF-C facilitates coordinated leading and lagging

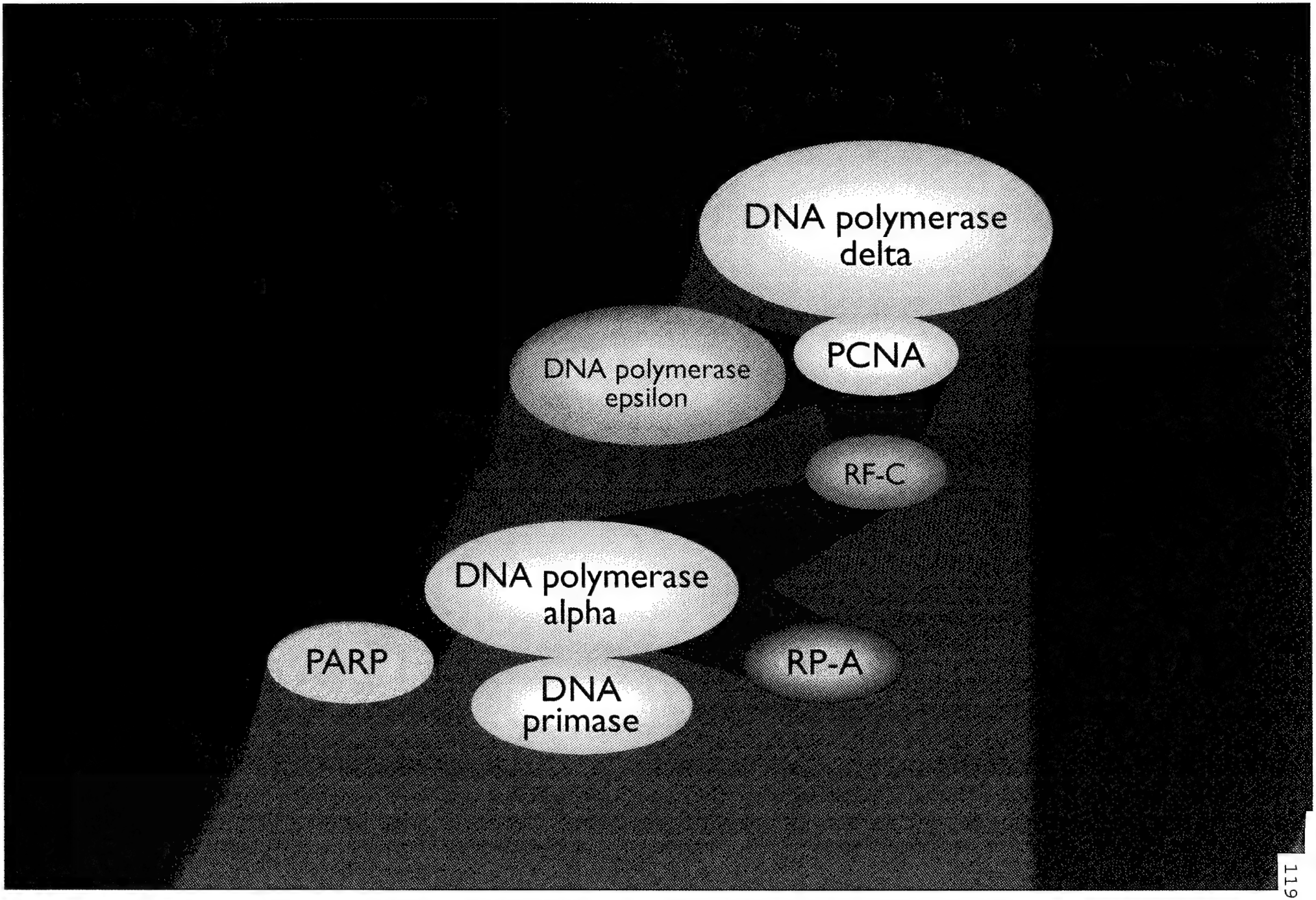

DNA polymerase delta
PCNA
DNA polymerase epsilon
RF-C
DNA polymerase alpha
PARP
RP-A
DNA primase

Figure 8

Figure 8. A model for the core component of the human breast cell DNA synthesome.

strand synthesis by serving as a molecular hinge between DNA polymerases α and δ. Our data support this hypothesis as we have provided direct evidence for a close association between DNA polymerases α, δ and RF-C within the breast cell DNA synthesome (Figure 8). Additionally, PCNA may participate in the coordination of leading and lagging strand synthesis by the DNA synthesome, as it was found to tightly associate with DNA polymerases α, δ and RF-C (Figure 8). Such a role for PCNA in DNA replication is supported by studies demonstrating that only early replicative lagging strand products are synthesized *in vitro* in the absence of the protein (39). Furthermore, as DNA polymerase ε represents a core component of the breast cell DNA synthesome and closely associates with PCNA in the complex, it may play a role in breast cancer cell DNA replication. Further work must be performed in order to determine the exact function of polymerase ε within the breast cell DNA synthesome. However, it has been proposed that the protein may mediate the conversion of DNA primers into Okazaki pieces (40) or act as a molecular sensor of DNA damage in eukaryotic cells (41).

We are currently conducting experiments to determine the protein members with which DNA ligase I as well as DNA topoisomerases I and II associate in the DNA synthesome. We believe that the complete characterization of the DNA synthesome will advance our understanding of the mechanisms and regulation of human breast cancer cell DNA replication. The observation that PARP modulates the *in vitro* replication activity of the DNA synthesome (36) and physically associates with DNA polymerase α in the complex (32), as demonstrated in this report, strongly suggests that PARP may serve to regulate replication initiation events mediated by the breast cell DNA synthesome. Determining the precise mechanism(s) by which PARP regulates the activity of the DNA

synthesome may facilitate the development of new anti-breast cancer agents capable of specifically turning off the replication activity of the complex.

ACKNOWLEDGMENTS

We would like to thank Drs. Christopher Mathews, G. Prem Veer Reddy, Ronald Berezney and Robert Freund for their critical review of the manuscript.

REFERENCES

1. Cropp, C.S. Frequent mutations in breast cancer. Cancer Lett. **90**: 51-56; 1995.

2. Hartwell, L.H. and Kastan, M.B. Cell cycle control and cancer. Science **266**: 1821-1828; 1994.

3. Keshgegian, A.A. and Cnaan, A. Proliferation markers in breast carcinoma. Am. J. Clin. Pathol. **104**: 42-50; 1995.

4. Mathews, C.K. and Slabaugh, M.B. Eukaryotic DNA metabolism: Are deoxyribonucleotides channeled to replication sites? Exp. Cell Res. **162**: 285-295; 1986.

5. Reddy, G. and Fager, R. Replitase: a complex integrating dNTP synthesis and DNA replication. Crit. Rev. Euk. Gene Exp. **3**: 255-277; 1993.

6. Hickey, R.J. and Malkas, L.H. Mammalian cell DNA replication. Crit. Rev. Euk. Gene Exp. **7**:125-157; 1997.

7. Tubo, R.A. and Berezney, R. Pre-replicative association of multiple replicative enzyme activities with the nuclear matrix during rat liver regeneration. J. Biol. Chem. **262**: 1148-1154; 1987.

8. Jackson, D.A. and Cook, P.R. Different populations of DNA polymerase α in HeLa cells. J. Mol. Biol. **192**: 77-86; 1986

9. Jackson, D.A. and Cook, P.R. Replication occurs at a nucleoskeleton. EMBO J. **5**: 1403-1410; 1986.

10. Malkas, L.H.; Hickey, R.J.; Li, C.J.; Pedersen, N.; Baril, E.F. A 21S enzyme complex from HeLa cells that functions in simian virus 40 DNA replication *in vitro*. Biochem. **29**: 6362-6374; 1990.

11. Applegren, N.; Hickey, R.J.; Kleinschmidt, A.M.; Zhou, Q.; Coll, J.M.; Wills, P.; Swaby, R.; Wei, Y.; Quan, J.Y.; Lee, M.Y.W.T.; Malkas, L.H. Further characterization of the human cell multiprotein DNA replication complex. J. Cell. Biochem. **59**: 91-107; 1995.

12. Wu, Y.; Hickey, R.J.; Lawlor, K.; Wills, P.; Yu, F.; Ozer, H.; Starr, R.; Quan, J-Y; Lee M.Y.W.T.; Malkas, L.H. A 17S multiprotein form of murine cell DNA polymerase mediates polyomavirus DNA replication *in vitro*. J. Cell. Biochem. **54**: 32-46; 1994.

13 Lin, S.; Hickey, R.J.; Malkas, L.H. The isolation of a DNA synthesome from human leukemia cells. Leuk. Res. **6**: 501-512; 1997.

14. Coll, J.M.; Sekowski, J.W.; Hickey, R.J.; Schnaper, L.; Yue, W.; Brodie, A.M.H.; Uitto. L.; Syvaoja, J.E.; Malkas, L.H. The human breast cell DNA synthesome: Its purification from tumor tissue and cell culture. Oncol. Res. **8**: 435-447; 1996.

15. Tom, T.; Hickey, R.J.; Malkas, L.H. Identification of multiprotein complexes containing DNA replication factors by native immunoblotting of HeLa cell protein preparations with T-antigen-dependent SV40 DNA replication activity. J. Cell. Biochem. **63**: 259-267; 1996.

16. Hurwitz, J.; Dean, F.B.; Kwong, A.D.; Lee, S.H. The *in vitro* replication of DNA containing the SV40 origin. J. Biol. Chem. **265**: 18043-18047; 1990.

17. Stillman, B. Smart machines at the DNA replication fork. Cell **78**: 725-728; 1994.

18. Kelman, Z. and O'Donnell, M. DNA polymerase III holoenzyme: structure and function of a chromosomal replicating machine. Annu. Rev. Biochem. **64**: 171-200; 1995.

19. Sambrook, J.; Fritsch, E.; Maniatis, T. Molecular cloning: A laboratory manual, 2nd edition, Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1989: pp E.l-E.39.

20. Stillman, B.; Gerard, R.D.; Guggenheimer, R.A.; Gluzman, Y. T-antigen and template requirements for SV40 DNA replication *in vitro*. EMBO J. **4**: 2933-2939; 1985.

21. Simanis, V. and Lane, D.P. An immunoaffinity purification procedure for SV40 large T-antigen. Virology **144**: 88-100; 1985.

22. LaMothe, P.; Baril, B.; Chi, A.; Lee, L.; Baril, E. Accessory proteins for DNA polymerase alpha activity with single-stranded templates. Proc. Natl. Acad. Sci. USA **78**: 4723-4727; 1981.

23. Vishwanatha, J.K.; Yamaguchi, M.; DePamphilis, M.L.; Baril, E.F. Selection of template initiation sites and the lengths of RNA primers synthesized by DNA primase are strongly affected by its organization in a multiprotein DNA polymerase alpha complex. Nucl. Acids Res. **14**: 7305-7323; 1986.

24. Tanaka, S.; Hu, S-Z; Wang, T.S.-F.; Korn , D. DNA polymerase-alpha: Common polypeptide core structure of three enzyme forms from human KB cells. J. Biol. Chem. **257**: 8386-8390; 1982.

25. Prelich. G.; Tan, C.K.; Mikostura, M.; Matthews, B.; So, A.G.; Downey, K.M.; Stillman, B. Functional identity of proliferating cell nuclear antigen and a DNA polymerase-delta auxiliary protein. Nature **326**: 517-520; 1987.

26. Brand, S.R.; Bernstein, R.M.; Mathews, M.B. Trimeric structure of human proliferating cell nuclear antigen: implication for enzymatic function and autoantibody recognition. J. Immunol., **15**: 3070-3079; 1994.

27. Nossal, N. Protein-protein interactions at a DNA replication fork: bacteriophage T4 as a model. FASEB **6**: 871-878; 1992.

28. Maga, G. and Hubscher, U. DNA replication machinery: functional characterization of a complex containing DNA polymerase alpha, DNA polymerase delta and replication factor C suggests an asymmetric DNA polymerase dimer. Biochem. **35**: 5764-5777; 1996.

29. Tsurimoto, T. and Stillman, B. Replication factors required for SV40 DNA replication *in vitro*. J. Biol. Chem. **266**: 1961-1968; 1991.

30. Eki, T. and Hurwitz, J. Influence of poly(ADP-ribose) polymerase on the enzymatic synthesis of SV40 DNA. J. Biol Chem. **266**: 3087-3100; 1991.

31. Dornreiter, I.; Erdile, L.F.; Gilbert, I.U.; von Winkler, D.; Kelly, T.J.; Fanning, E. Interaction of DNA polymerase alpha-primase with cellular replication protein A and SV40 T-antigen. EMBO J. **11**: 769-176; 1992.

32. Simbulan, C.M.G.; Suzuki, M.; Izuta, S.; Sakurai, T.; Savoysky, E.; Kojima, K.; Miyahara, K.; Shizuta, Y.; Yoshida, S. Poly(ADP-ribose) polymerase stimulates DNA polymerase alpha by physical association. J. Biol. Chem. **268**: 93-99; 1993.

33. Smulson, M.; Kang, V.; Ntambi, J.; Rosenthal, D.; Ding, R.; Simbulan, C. Requirement for the expression of poly(ADP-ribose) polymerase during the early stages of differentiation of 3T3-L1 preadipocytes, as studied by antisense RNA induction. J. Biol. Chem. **270**: 119-127; 1995

34. Yoshihara, K.; Itaya, A.; Tanaka, Y.; Ito, K.; Teraoka, H.; Tsukada, K.; Matsukage. A.; Kamiya, T. Inhibition of DNA polymerase alpha, DNA polymerase beta, terminal

deoxynucleotide transferase and DNA ligase II by poly(ADP-ribosyl)ation reaction *in vitro*. Biochem. Biophys. Res. Commun. **128**: 61-67; 1985.

35. Ferro, A.M. and Olivera, B. Poly(ADP-ribosyl)ation of DNA topoisomerase I from calf thymus. J. Biol. Chem. **259**: 547-554; 1984.

36. Simbulan-Rosenthal, C.M.; Rosenthal, D.S.; Hilz, H.; Hickey, R.J.; Malkas, L.H.; Applegren, N.; Wu, Y.; Bers, G.; Smulson, M.E. The expression of poly(ADP-ribose) polymerase during differentiation-linked DNA replication reveals that it is a component of the multiprotein DNA replication complex. Biochem. **35**: 11622-11633; 1996.

37. Waga, S. and Stillman, B. Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication *in vitro*. Nature **369**: 207-212; 1994.

38. Morris, G.F. and Mathews, M.B. Regulation of proliferating cell nuclear antigen during the cell cycle. J. Biol. Chem. **23**: 13856-13864; 1989.

39. Prelich, G. and Stillman, B. Coordinated leading and lagging strand synthesis during SV40 DNA replication *in vitro* requires PCNA. Cell **53**: 117-126; 1988.

40. Nethanel, T. and Kaufmann, G. Two DNA polymerases may be required for the synthesis of the lagging DNA strand of simian virus 40. J. Virology **64**: 5912-5918; 1990.

41. Zlotkin, T.; Kauffman, G.; Jiang, Y.; Lee, M.Y.W.T.; Uitto, L.; Syvaoja, J.E.; Dornreiter, I.; Fanning, E.; Nethanel, T. DNA polymerase epsilon may be dispensable for SV40, but not cellular DNA replication. EMBO J. **15**: 2298-2305; 1996.

Chapter 4

The Breast Cell DNA Synthesome: A Novel Model for Examining the Mechanisms of Action of Irinotecan and Etoposide

Jennifer M. Coll [(1)], Erica A. Cronkey [(1)], Robert J. Hickey [(1-4)], Lauren Schnaper [(5,6)] and Linda H. Malkas [(1-4) *]

[1]Department of Pharmacology and Experimental Therapeutics, [2]Program in Molecular and Cellular Biology, [3]Program in Oncology, [4]Program in Toxicology, [5]Department of Surgery, University of Maryland School of Medicine, Baltimore, MD 21201; [6]Breast Evaluation and Treatment Center, Greater Baltimore Medical Center, Baltimore, MD 21240.

Running Title: A Novel Model for Examining the Mechanisms of Action of SN-38 and VP-16

Key Words: DNA replication; anticancer agent; etoposide; irinotecan; multiprotein complex; breast cancer; synthesome; topoisomerase

ABSTRACT

We have previously described the isolation and characterization of an intact multiprotein complex for DNA replication, designated the DNA synthesome, from human breast cancer (MDA MB-468) cells and biopsied human breast tumor tissue. In the presence of the viral large T-antigen, the breast cell DNA synthesome fully supports the replication of simian virus 40 (SV40) origin-containing DNA *in vitro.* Moreover, as shown in this report, the daughter DNA molecules produced by the breast cell DNA synthesome consist of monomeric, closed circular form I DNAs as well as topological and replicative intermediates that are resistant to digestion by *Dpn*I; which is consistent with the criteria for semi-conservative replication. As the DNA synthesome represents the intact breast cell's DNA replication machinery, it may serve as a novel model for examining the mechanisms of action of anti-breast cancer agents that target the DNA synthetic process. In this report, we present for the first time data that indicate the breast cell DNA synthesome serves as a powerful model for studying the actions of irinotecan (CPT-11) and etoposide (VP-16)--two anti-breast cancer agents that selectively target topoisomerases I and II, respectively. We found a close correlation between the IC_{50} values for the inhibition of intact cell and DNA synthesome-mediated *in vitro* DNA replication by CPT-11 (SN-38) (1.5 μM and 0.2 μM, respectively) and VP-16 (2 μM and 0.5 μM, respectively). Additionally, we found that similar concentrations of SN-38 and VP-16, 0.5 μM each, inhibit topoisomerase I and II enzymatic activities by 50% as well as produce significant levels of cleavable complexes. Consistent with these findings, alkaline agarose gel electrophoretic analysis of the DNA molecules synthesized *in vitro* by the

DNA synthesome in the presence of SN-38 or VP-16 (0.2 μM and 0.5 μM, respectively) indicates that both agents cause an extensive accumulation of short daughter DNA molecules. Ultimately, utilization of the breast cell DNA synthesome as a model for studying the mechanisms of action of SN-38 and VP-16 may provide clearer insight into their modes of action and aid the development of improved analogues of these agents for breast cancer treatment.

Title Footnote:

***Author: Linda H. Malkas,** author to whom correspondence should be addressed

Address: University of Maryland School of Medicine
(1) Department of Pharmacology and Experimental Therapeutics
(3) Program in Molecular and Cellular Biology
(4) Program in Oncology
(5) Program in Toxicology
685 W. Baltimore St.
Baltimore, MD 21201

Tel: (410) 706-2313 or 1798
Fax: (410) 706-0337

Author: Jennifer M. Coll

Address: University of Maryland School of Medicine
(1) Department of Pharmacology and Experimental Therapeutics

Author: Erica A. Cronkey

Address: University of Maryland School of Medicine
(1) Department of Pharmacology and Experimental Therapeutics

Author: Robert J. Hickey

Address: University of Maryland School of Medicine
(1) Department of Pharmacology and Experimental Therapeutics
(2) Program in Molecular and Cellular Biology
(3) Program in Oncology
(4) Program in Toxicology

Author: Lauren Schnaper

Address: Greater Baltimore Medical Center
(6) Comprehensive Breast Evaluation Center
(7) Department of Surgery

Abbreviations used: SV40, simian virus 40; CPT-11, irinotecan; VP-16, etoposide; CPT, camptothecin; PCNA, proliferating cell nuclear antigen; RP-A, replication protein A; RF-C, replication factor C; PARP, poly(ADP-ribose) polymerase; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; K^+, potassium.

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women in the United States [1]. Although the incidence of breast cancer continues to rise annually, it is promising that both primary and adjuvant therapy of the disease improve survival in pre and post-menopausal patients [2-5]. Many of the anticancer agents that demonstrate efficacy in the treatment of breast neoplasms work during active cell division to modulate the processes of DNA synthesis, transcription or mitotic tubule spindle formation [6]. For example, CPT-11 (active metabolite: SN-38) and VP-16 are potent inhibitors of DNA replication that have shown significant activity against breast cancer in clinical trials and are currently being utilized in multi-drug chemotherapy regimens against the disease [7-9].

Specifically, CPT-11 and VP-16 inhibit breast cancer cell DNA replication by trapping DNA topoisomerases I and II, respectively, in ternary (drug-enzyme-DNA) cleavable complexes [10-14]. In this state, neither DNA topoisomerase I nor II can perform its DNA nicking-resealing function necessary for the relaxation of topological constraints on DNA. It has been postulated that the interaction of CPT-11-stabilized topoisomerase I DNA cleavable complexes with advancing replication forks during the initiation and elongation stages of DNA synthesis leads to irreparable DNA damage and cytotoxicity [13,15,16]. However, the mechanisms by which the production of VP-16-stabilized topoisomerase II DNA cleavable complexes induce cell death remain unclear.

A few experimental methods have been utilized to a great extent to assess the mechanisms of action of anti-cancer agents that directly target DNA synthesis. These approaches include examining the effects of anti-cancer agents on SV40 DNA replication

in vitro reconstituted with purified proteins or mediated by crude cell extracts as well as studying the interactions of the agents with their purified target DNA replication proteins [15,17-20]. Although the use of these approaches has provided important insights into the mechanisms of action of several anti-breast cancer agents, each method possesses serious limitations. First, not all of the proteins participating in DNA synthesis have been discovered; so, *in vitro* SV40 DNA replication systems reconstituted with purified proteins may lack important components of the cellular DNA replication machinery. Second, the use of *in vitro* SV40 DNA replication mediated by crude cell extracts as a drug model does not permit the assessment of drug action in the absence of other enzymatic processes that may secondarily affect DNA synthesis. Third, the interaction of an anticancer agent with its target protein may be quite different when the protein associates with other components of the DNA replication machinery as opposed to when it acts alone in the presence of drug. These methodological limitations are underscored by reports of significant discrepancies between the concentrations of agents required to inhibit *in vitro* SV40 DNA replication activity or purified target protein function versus intact cell DNA synthesis [21-23]. Indeed, the use of a model which represents the DNA replication machinery of intact breast cells would greatly facilitate the study of the mechanisms of action of many anti-breast cancer agents.

We have previously described the isolation and characterization of a multiprotein complex for DNA synthesis, designated the DNA synthesome, from several human cell lines [24-28], including human breast cancer (MDA MB-468) cells as well as from biopsied human breast tumor tissue [29]. The proteins and enzymes found to copurify with

the DNA synthesome include: DNA polymerases α, δ, and ε, DNA primase, PCNA, RF-C, RP-A, DNA helicases I and IV, DNA ligase I, PARP and DNA topoisomerases I and II [24-29]. We have shown that the integrity of the DNA synthesome is maintained after its treatment with salt, detergent, RNase, DNase, anion-exchange chromatography, sedimentation through glycerol or sucrose gradients and electrophoresis through native polyacrylamide gels [24-30]. Additionally, we have recently reported that several of the core components of the DNA synthesome are co-precipitated by antibodies directed against DNA polymerases α, δ and PCNA [31]. Overall, these results indicate that the DNA synthesome represents a complex of tightly associated replication proteins and that the interactions of the protein components with one another is independent of non-specific interactions with other cellular macromolecules.

Most importantly, in the presence of the viral large T-antigen and SV40 origin sequences, the DNA synthesome fully supports semi-conservative SV40 DNA replication *in vitro* [32]. The requirements for DNA synthesome mediated replication *in vitro* are comparable to those observed for cells permissive for SV40 infection [33], strongly suggesting that the DNA synthesome represents the intact cell's DNA replication machinery. As such, the breast cell DNA synthesome may serve as a novel model for examining the mechanisms of action of anti-breast cancer agents that directly target DNA synthesis.

In this report, we provide evidence that the breast cell DNA synthesome represents a novel model for examining the mechanisms of action of SN-38 and VP-16. Ultimately, a

clearer understanding of the modes of action of SN-38 and VP-16 may facilitate the development of improved analogues of these agents for breast cancer treatment.

MATERIALS AND METHODS

Materials. CPT and VP-16 were purchased from Sigma Chemical Co. (St. Louis, MO). CPT-11 and its active metabolite, SN-38, were kindly supplied by Dr. John Wilkes of Pharmacia/UpJohn (Kalamazoo, MI). All drugs were dissolved in DMSO at stock concentrations of 5.0 mM and stored at -20°C.

Purified DNA topoisomerases I (2 units/μl) and II (2 units/μl) were purchased from TopoGen, Inc. (Columbus, Ohio). One unit of topoisomerase I relaxes 250 ng supercoiled DNA in 30 minutes at 37°C; while, one unit of topoisomerase II decatenates 200 ng *Crithidia fasciculata* K DNA networks in 30 minutes at 37°C.

α[^{32}P]dCTP and α[^{32}P]dATP (3000 Ci/mmol; 370 MBq/ml; 10 mCi/ml) as well as [^{3}H]-thymidine (90 Ci/mmol; 37 MBq/ml; 2.5 mCi/ml) were obtained from DuPont New England Nuclear (Boston, MA).

Methods

Cell Culture. Suspension cultures of MDA MB-468 human breast carcinoma cells were adapted from monolayer cultures. The cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of new-born calf serum and FBS. Exponentially growing cells ($5xl0^5$ cells/ml of medium) were harvested and washed three times with PBS: 20 mM Na_2HP0_4, 0.15 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4. The cells were then pelleted by low-speed centrifugation (1000 rpm, 5 minutes, 4°C), and the cell pellets stored at -80°C until fractionation.

Isolation and purification of the DNA synthesome from MDA MB-468 breast cancer cells. MDA MB-468 breast cancer cells (15 g) were homogenized and the breast cell DNA synthesome was purified as described in our previously published procedures [29]. The protein fraction designated the Q-Sepharose (Pharmacia, Piscataway, NJ) peak (0.9 μg/μl), which contains the replication-competent DNA synthesome, was used in the experiments described in this report.

Measurement of intact MDA MB-468 cell DNA synthesis. Exponentially growing MDA MB-468 breast cancer cells ($5x10^4$ per dish) were incubated for 1 hr at 37°C with [^{3}H]-thymidine (1 μCi/ml tissue culture media) and exposed to either CPT-11, CPT or VP-16 freshly prepared in DMSO. Controls in which cells were exposed to DMSO alone were also performed. Following the 1 hr incubation, cells were washed once with warm tissue-culture media then three times with cold PBS. The cells were then lysed and the amount of radiolabel incorporated into DNA was determined by the isolation and counting of acid-insoluble material [34].

***In vitro* SV40 DNA replication assay**. Assay reaction mixtures (12.5 μl) contained 80 mM Tris-HCl, pH 7.5; 7 mM $MgCl_2$; 1 mM DTT; 5-10 μg Q-Sepharose peak; 0.5-1.0 μg purified SV40 large T-antigen; 25 ng plasmid $pSVO^+$ containing an insert of SV40 replication-origin DNA sequences [35]; 100 μM each dTTP, dATP, dGTP; 200 μM each rCTP, rGTP, rUTP; 4mM ATP; 50 μM α[^{32}P]-dCTP; 40 mM creatine phosphate; 1μg creatine phosphokinase. The standard reaction, conducted in the absence or presence of SN-38, CPT or VP-16, was incubated for 2 hr at 37°C. Replication assay products were processed using DE81 filter binding to determine the amount of radiolabel incorporated

into acid insoluble material [35]. One unit of SV40 replication activity is equivalent to the incorporation of 1 pmol dCMP into newly synthesized DNA per 1h under the standard assay conditions. For neutral and alkaline agarose gel electrophoretic analyses, DNA replication assays (25 μl) were performed for 1 hr and the DNA products were processed as described in a later section of these Materials and Methods.

DNA topoisomerase I assay. DNA synthesome-associated topoisomerase I activity was measured by incubating 150 ng supercoiled $pSVO^+$ DNA with 8 μg Q-Sepharose peak in a buffer containing: 10 mM Tris-HCl (pH 7.9); 1 mM EDTA; 0.15 M NaCl; 0.1% BSA; 0.1 mM spermidine and 5% glycerol. To examine the effects of SN-38 on DNA synthesome-associated topoisomerase I activity, reactions were also performed in the presence of this agent. Reactions were incubated for 15 minutes at 37°C. Each reaction (20 μl) was stopped by the addition of 2 μl 10% SDS. The DNA products were then resolved at 40 volts on a 1.0 % agarose gel containing TAE buffer (40 mM Tris-acetate, 2 mM EDTA). After ethidium bromide (1 μg/ml) staining of the gel [35], topoisomers were visualized by illuminating the gel with an ultraviolet light source. To assess the percent inhibition of synthesome-associated topoisomerase I activity by SN-38, the relative densities of form I supercoiled DNA contained in lanes 1-5 (Figure 1) were quantitated with a Molecular Dynamics Personal Densitometer SI and compared to the density of supercoiled DNA contained in lane 6 (Figure 1).

DNA topoisomerase II assay. DNA synthesome-associated topoisomerase II activity was measured by incubating 260 ng of *Crithidia fasciculata* K DNA networks with 8 μg Q-Sepharose peak in a buffer containing: 50 mM Tris-HCl (pH 8.0); 120 mM KCl; 10

mM $MgCl_2$; 0.5 mM ATP; 0.5 mM DTT; 30 μg/ml BSA. To examine the effects of VP-16 on DNA synthesome-associated topoisomerase II activity, reactions were also performed in the presence of the agent. Reactions were incubated for 30 minutes at 37°C. Each reaction (20 μl) was stopped by adding EDTA to a final concentration of 25 mM; protein was then digested with 50 ng/μl of proteinase K for an additional 15 minutes at 37°C. After the addition of gel loading buffer and the extraction of DNA with chloroform/isoamyl alcohol, the DNA products were resolved at 80 volts on a 1% agarose gel containing TBE buffer (50 mM Tris-borate; 1 mM EDTA) and ethidium bromide (0.5 μg/ml). The DNA was visualized by illuminating the gel with an ultraviolet light source. To assess the percent inhibition of topoisomerase II activity by VP-16, the relative densities of the decatenated, monomeric DNAs contained in lanes 2-6 (Figure 2) were quantitated with a Molecular Dynamics Personal Densitometer SI and compared to the density of decatenated DNA contained in lane 1 (Figure 2).

Quantitation of the amount of topoisomerases I and II present in the Q-Sepharose peak. One unit of DNA topoisomerase I activity is sufficient to convert fully 250 ng supercoiled, form I DNA into open-circle, form II DNA in 30 minutes at 37°C. The minimal amount of DNA synthesome-associated topoisomerase I necessary to convert fully 250 ng supercoiled, form I DNA to open-circular, form II DNA was determined by performing topoisomerase I assays with dilutions of the Q-Sepharose peak (data not shown). Using this approach, we estimated that 8 μl (8 μg) of the Q-Sepharose peak contains one unit of DNA topoisomerase I activity. The amount of DNA topoisomerase II present in the Q-Sepharose peak was determined by the method described above (data not

shown), where one unit of topoisomerase II activity is sufficient to decatenate 200 ng K DNA networks in 30 minutes at 37°C. We estimated that 8 µl of the Q-Sepharose peak contains one unit of DNA topoisomerase II activity.

3' end-labeling of pSVO$^+$ plasmid DNA. 10 µg pSVO$^+$ plasmid DNA was incubated with 20 units EcoRI restriction endonuclease (New England BioLabs, Boston, MA) in 1X EcoRI buffer overnight at 37°C. The cut DNA (10 µg) was incubated with 5 units Klenow fragment (Stratagene, LaJolla, CA) in the presence of 5 mM each dATP and TTP and 80 µCi $\alpha[^{32}P]$dATP for 1 hr at 37°C. Reactions were stopped by adding EDTA to a final concentration of 25 mM; the reactions were then diluted to 70 µl with STE buffer containing: 0.1 M NaCl; 10 mM Tris-HCl (pH 8.0); and 1 mM EDTA. Unincorporated deoxynucleoside triphosphates were removed by chromatography through a P60 gel filtration column [34].

SDS precipitation of DNA synthesome-associated topoisomerase I and II cleavable complexes. Cleavage of DNA by topoisomerases I and II was performed as follows. Briefly, 10 µg Q-Sepharose peak was incubated with 220 ng 3' end-labeled pSVO$^+$ DNA in topoisomerase I or II reaction buffer. Assays were conducted in the absence or presence of either SN-38 or VP-16. Reactions (30 µl) were incubated for 30 minutes at 37°C then stopped by the addition of 3 µl 10% SDS and 250 µl buffer A1 (TopoGen Inc., Columbus, OH) containing: 10 mM Tris-HCl (pH 7.5); 20 µg/ml BSA; 20 µg/ml calf thymus DNA and 1% SDS. Double-stranded topoisomerase-DNA cleavable complexes were precipitated by KCl (added to a final concentration of 0.2 M) followed by incubation on ice for 15 minutes. The precipitated topoisomerase-DNA cleavable complexes were

isolated by filtration onto glass fiber filters, and processed according to the manufacturer's guidelines (TopoGen Inc., Columbus, OH).

Neutral and alkaline gel electrophoretic analyses of the DNA products synthesized by the DNA synthesome. *In vitro* DNA replication assays were performed in the presence of either CPT, SN-38 or VP-16 at their IC_{50} concentrations for the inhibition of synthesome mediated DNA synthesis. For both neutral and alkaline agarose gel electrophoretic analyses of DNA products, the DNA replication reactions (25 μl) were terminated by the addition of EDTA to a final concentration of 25 mM, SDS to a final concentration of 1% and 50 μg yeast tRNA as a carrier. Next, the protein contained in the DNA replication reactions was digested with 50 ng/μl proteinase K for 1 hr at 37°C. The DNA products were then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (24:25:1), followed by chloroform/isoamyl alcohol (24:1). The DNA products were precipitated by 2.0 M ammonium acetate and 100% ethanol, then washed three times with 70% ethanol. For neutral agarose gel electrophoresis, the DNA pellets were resuspended in 20 μl TE buffer: 10 mM Tris (pH 7.4) and 0.1 mM EDTA (pH 8.0). After the addition of gel loading buffer, the DNA products were loaded onto a 1.2% agarose gel containing TBE buffer and resolved overnight at 30 volts. For analysis of denatured DNA products, the DNA pellets were resuspended in 15 μl of a buffer containing 50 mN NaOH and 1 mM EDTA. After the addition of alkaline gel loading buffer, the DNA products were loaded onto a 1% alkaline agarose gel containing 50 mN NaOH and 1 mM EDTA and resolved overnight at 30 volts. Following electrophoresis, the resolved DNA products

were fixed with 7% TCA. Both the neutral and alkaline agarose gels were dried under vacuum and exposed to Kodak XAR-5 film at –80°C.

RESULTS

Inhibition of intact cell and DNA-synthesome mediated in vitro DNA replication by CPT-11 (SN-38) and VP-16

To demonstrate that CPT-11, its parent compound, CPT, and VP-16 are potent inhibitors of cellular DNA replication, we measured the level of DNA synthesis in intact MDA MB-468 breast cancer cells exposed to increasing concentrations of these agents. Briefly, exponentially growing MDA MB-468 breast cancer cells were exposed for 1 hr to logarithmically increasing concentrations of either CPT-11, CPT or VP-16 in the presence of [^{3}H]-thymidine. Afterward, cellular DNA replication activity was determined by quantitating the amount of [^{3}H]-TMP incorporated into acid-insoluble material (Materials and Methods). The exposure of breast cancer cells to increasing concentrations of either CPT-11, CPT or VP-16 resulted in a dose-dependent decrease in [^{3}H]-TMP incorporation into cellular DNA (data not shown). DNA replication activity was inhibited 50% by 1.5 μM CPT-11 or 0.05 μM CPT. These results are consistent with previously published results regarding the effects of CPT-11 and CPT on intact mouse leukemia (P388) cell DNA synthesis [36]. Also, we found that 2 μM VP-16 inhibits intact MDA MB-468 cell DNA synthesis by 50% (Table 1); which is consistent with findings reported by Tadou et al. regarding the inhibitory effects of VP-16 on intact mouse splenocyte DNA replication [37].

To determine whether SN-38, the active metabolite of CPT-11, and VP-16 inhibit DNA synthesome-mediated *in vitro* DNA synthesis, we performed *in vitro* DNA

Table 1. IC_{50} values for the inhibition of intact MDA MB-468 cell DNA synthesis and DNA replication *in vitro* by SN-38, CPT and VP-16.

Anticancer Agent	Intact Cell DNA Synthesis[+]	DNA Synthesis *In Vitro*[*]
CPT-11/SN-38	1.5 μM (R=0.96)	0.25 μM (R=0.96)
CPT	0.05 μM (R=0.9)	0.5 μM (R=0.95)
VP-16	2.0 μM (R=0.9)	0.5 μM (R=0.95)

[+] Experiments to measure MDA MB-468 intact cell DNA synthesis were performed in the presence of increasing concentrations of SN-38, CPT or VP-16 (0.05-500 μM). Standard curves for the inhibition of DNA synthesis by these agents were generated and used to determine IC_{50} values. Each point on the curves had a minimum n of 2.

[*] *In Vitro* DNA replication assays were performed in the presence of SN-38 or VP-16 (0.05-500 μM). Standard curves for the inhibition of *in vitro* DNA synthesis by SN-38 or VP-16 were generated and used to determine IC_{50} values. Each point on the curves had a minimum n of 2.

replication assays (Materials and Methods) in the presence of increasing concentrations of these agents. Additionally, as a control, we performed *in vitro* DNA replication assays in the presence of increasing concentrations of CPT, as we have previously demonstrated that this agent strongly inhibits DNA synthesis mediated by the HeLa cell synthesome [38]. We found that 0.2 μM SN-38 and 0.5 μM CPT inhibit the incorporation of [^{32}P]-dCMP into newly synthesized DNA by 50% (Table 1). In addition, we found that 0.5 μM VP-16 inhibits *in vitro* DNA synthesis by 50% (Table 1). These IC_{50} values for *in vitro* DNA replication mediated by the DNA synthesome correlate closely with those concentrations of CPT-11, CPT and VP-16 inhibiting MDA MB-468 intact cell DNA synthesis by 50%. Overall, these results are consistent with the hypothesis that the DNA synthesome represents the intact breast cell's DNA replication machinery.

Effects of SN-38 and VP-16 on DNA synthesome-associated topoisomerase I and II activities

Topoisomerase I relaxes positively supercoiled DNA by introducing a transient single-strand nick into the DNA phosphodiester backbone, facilitating strand passage. We performed topoisomerase I assays to demonstrate that the topoisomerase I activity present in the DNA synthesome is fully able to produce the hallmark ladder of DNA intermediates while converting a form I supercoiled plasmid DNA to a relaxed, open-circular form II DNA. As shown in Figure 1 (lanes 7 and 8), the pattern of topoisomers produced by the DNA synthesome-associated topoisomerase I is indistinguishable from that generated by the purified enzyme. Furthermore, we estimated that the level of topoisomerase I present in the Q-Sepharose peak is approximately 1 unit/8 μg of synthesome protein (Materials

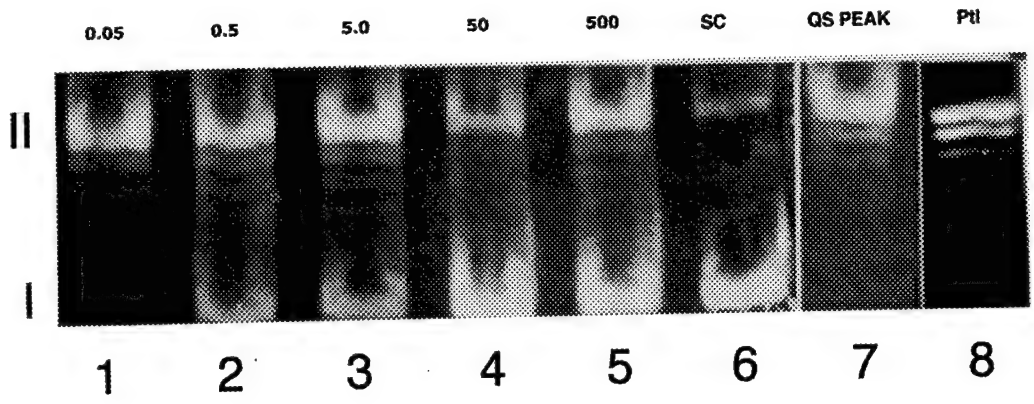


Figure 1. Inhibition of DNA synthesome-associated topoisomerase I activity by SN-38. The Q-Sepharose peak (8 μg) was incubated with 150 ng of supercoiled pSVO$^+$ DNA for 30 minutes at 37°C in the presence of increasing concentrations of SN-38 (0.05, 0.5, 5, 50, 500 μM) dissolved in DMSO. Reactions containing DMSO alone served as positive controls (lane 7). The reactions (20 μl) were stopped by the addition of SDS to a final concentration of 1%; and the protein was digested with proteinase K (50 ng/μl) for fifteen minutes at 37°C. Afterward, the DNA topoisomers were resolved by electrophoresis (45 volts) through a 1% agarose gel containing TAE buffer (Materials and Methods). Following electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) and the topoisomers were visualized by illuminating the gel with an ultraviolet light source. Lane 6 shows the position of supercoiled pSVO$^+$ DNA. Lanes 7 and 8 show the conversion of supercoiled, form I DNA into relaxed, open-circular form II DNA by the DNA synthesome-associated and purified topoisomerase I enzymes, respectively. Lanes 1-5 show the concentration dependent inhibition of DNA synthesome-associated topoisomerase I activity by SN-38 (0.05-500 μM, respectively).

and Methods). We performed topoisomerase I assays in the presence of increasing concentrations of SN-38 to examine the effects of the agent on DNA synthesome-associated topoisomerase I activity (Materials and Methods). In this assay, the inhibition of topoisomerase I activity by SN-38 prevents the full conversion of form I supercoiled plasmid DNA to open circle form II DNA. We observed an extensive level of inhibition of the DNA synthesome-associated topoisomerase I activity by as little as 0.5 μM SN-38 (Figure 1, lane 2). By comparing the relative densities of form I supercoiled DNA contained in lanes 2 and 6, we determined that 0.5 μM SN-38 inhibits DNA synthesome-associated topoisomerase I activity just over 50% (Materials and Methods). This concentration of SN-38 is comparable to that inhibiting *in vitro* DNA synthesis by 50%. This close correlation between IC_{50} values supports the use of the DNA synthesome as a drug model, as SN-38 inhibits DNA replication by selectively targeting topoisomerase I activity.

We also performed topoisomerase II assays to demonstrate that the DNA synthesome contains a fully functional topoisomerase II. Similar to the purified enzyme (Figure 2, lane 7), the DNA synthesome-associated topoisomerase II is fully able to decatenate *Crithidia fasciculata* kinetoplast DNA (K DNA) to generate monomeric, open-circular DNAs in an ATP dependent reaction (Figure 2, lane 1). Moreover, we have previously shown that in reaction assays lacking ATP, the DNA synthesome-associated topoisomerase II does not support the decatenation of K DNA [29]. We estimated that the level of topoisomerase II present in the Q-Sepharose peak is approximately 1 unit/8 μg of synthesome protein (Materials and Methods).

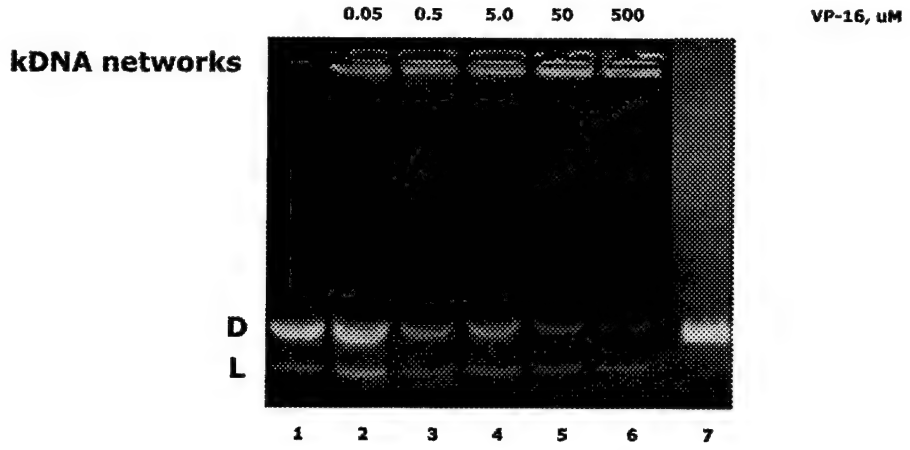


Figure 2. Inhibition of DNA synthesome-associated topoisomerase II activity by VP-16. The Q-Sepharose peak (8 μg) was incubated with 260 ng of K DNA for 45 minutes at 37°C in the presence of increasing concentrations of VP-16 (0.05, 0.5, 5, 50, 500 μM) dissolved in DMSO. Reactions containing DMSO alone served as positive controls (lane 1). The reactions were stopped by the addition of 2 μl 0.5 M EDTA and SDS to a final concentration of 1%. After proteinase K digestion (50 ng/μl) and the addition of gel loading buffer to the samples, the DNA products were extracted once with chloroform/isoamyl alcohol. The DNA products were resolved at 100 volts on a 1% agarose gel containing TBE buffer (Materials and Methods) and ethidium bromide (0.5 μg/ml). Following electrophoresis, the DNA products were visualized by illuminating the gel with an ultraviolet light source. The positions of decatenated and linear DNAs are designated by D and L, respectively. Lanes 1 and 7 show the relaxation of K DNA to decatenated monomers (D) by synthesome-associated and purified topoisomerase II enzymes, respectively. Lanes 2-6 show the concentration dependent inhibition of DNA synthesome-associated topoisomerase II activity by VP-16 (0.05-500 μM).

We next examined the effects of increasing concentrations of VP-16 on DNA synthesome-associated topoisomerase II activity. At 0.5 μM VP-16, the DNA synthesome-associated topoisomerase II was significantly inhibited from supporting the decatenation of K DNA networks to open-circular, monomeric DNA (Figure 2, lane 3). By comparing the relative densities of the decatenated, monomeric DNAs between lanes 1 and 3, we found that 0.5 μM VP-16 inhibited synthesome-associated topoisomerase II activity greater than 50% (Materials and Methods). This result is consistent with the premise that VP-16 inhibits DNA synthesis by targeting topoisomerase II activity.

DNA Topoisomerase I and II Cleavable Complex Stabilization by SN-38 and VP-16

SN-38 and VP-16 interfere with the DNA breakage-reunion activities of topoisomerases I and II, respectively, by trapping the enzymes in reversible ternary (drug-enzyme-DNA) cleavable complexes [36,39]. In these states, topoisomerases I and II remain covalently attached to the ends of nicked substrate DNA, unable to catalyze the religation of DNA strand breaks [12]. It has been shown that cleavable complexes can be precipitated by a strong protein denaturant, like SDS, and high concentrations of potassium (K^+) [19,39]. In this type of assay, the addition of SDS and K^+ to reaction tubes causes the precipitation of sample proteins as well as the co-precipitation of any DNA covalently bound to protein. Therefore, utilizing 3' end-labeled substrate DNA, the relative amount of topoisomerase-DNA cleavable complexes stabilized by SN-38 and VP-16 can be quantified by determining the amount of co-precipitated, radiolabeled DNA. We performed SDS/K^+ precipitation assays using the Q-Sepharose peak fraction to determine the relative amount of synthesome-associated topoisomerase DNA cleavable complexes

stabilized by SN-38 and VP-16. We found that at low concentrations of these agents (0.05 and 0.5 μM), significant levels of cleavable complexes were formed compared to control reactions performed in the absence of drug (Figure 3). The formation of significant levels of synthesome-associated topoisomerase DNA cleavable complexes at low concentrations of SN-38 and VP-16 is consistent with our observation that 0.2 μM and 0.5 μM of these agents, respectively, inhibit *in vitro* DNA replication activity by 50%.

Neutral and Alkaline Agarose Gel Electrophoretic Analyses of the DNA Products Synthesized by the DNA Synthesome in the Presence of SN-38 and VP-16

To examine more closely the effects of SN-38, as well as CPT, and VP-16 on DNA synthesome-mediated *in vitro* DNA replication, we subjected the DNA products synthesized by the complex in the presence of these agents to alkaline agarose gel electrophoresis. The DNA products synthesized by the synthesome in positive control reactions ranged in size from less than 100 nucleotides to two full-length products (single-stranded linear [ssl] and single stranded circular [ssc]) (Figure 4, lane 3). Moreover, resolution of these daughter DNA molecules on neutral agarose gels demonstrates that the majority of products consist of monomeric form I DNAs as well as topological and replicative intermediates that are resistant to digestion by *Dpn*I (Figure 4, lane 1); which is consistent with the criteria for semi-conservative DNA replication *in vitro.* Additionally, DNA synthesome-mediated replication *in vitro* is large T-antigen dependent, as no DNA products were formed in the absence of the viral protein (Figure 4, lane 2). In the presence of 0.2 μM SN-38, 0.5 μM CPT or 0.5 μM VP-16, those concentrations determined to be

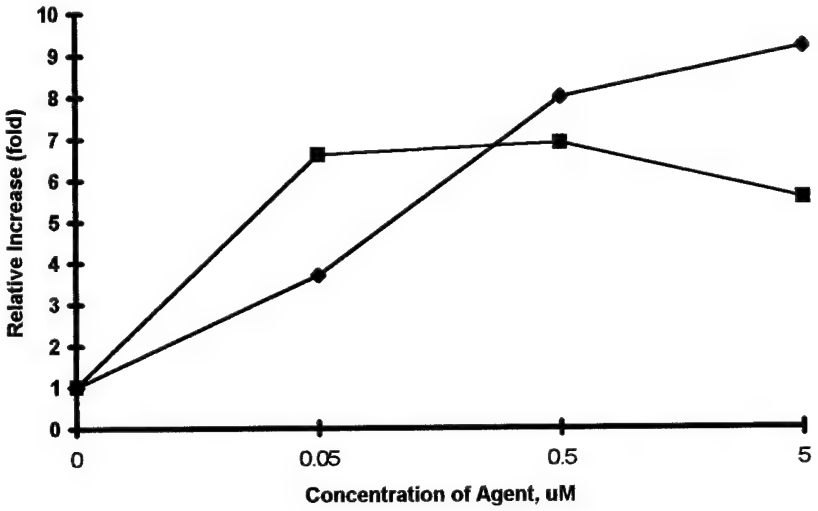


Figure 3. SDS-K^+ precipitation of SN-38 and VP-16 stabilized topoisomerase DNA cleavable complexes. Reaction assays containing 10 μg Q-Sepharose peak, 220 ng 3'-end labeled $pSVO^+$ DNA and increasing concentrations of either SN-38 (■) or VP-16 (◆) (0.05-5 μM), dissolved in DMSO, were incubated for 30 minutes at 37°C. Reactions containing DMSO alone served as controls. The reactions (30 μl) were stopped by the addition of 3 μl 10% SDS and 250 μl of buffer A1 (Materials and Methods). Following their precipitation by KCl, the topoisomerase DNA covalent cleavable complexes were isolated by vacuum filtration onto glass fiber filters, and the filters were washed and counted in a liquid scintillation spectrophotometer. The curves shown are representative of those obtained in these assays.

Figure 4, panel 1

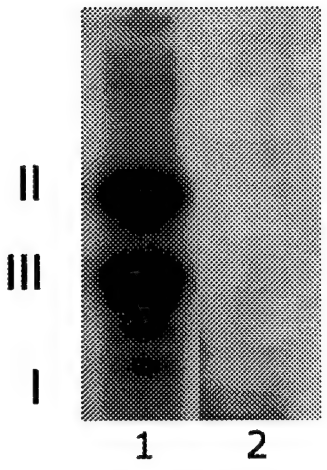


Figure 4, panel 2

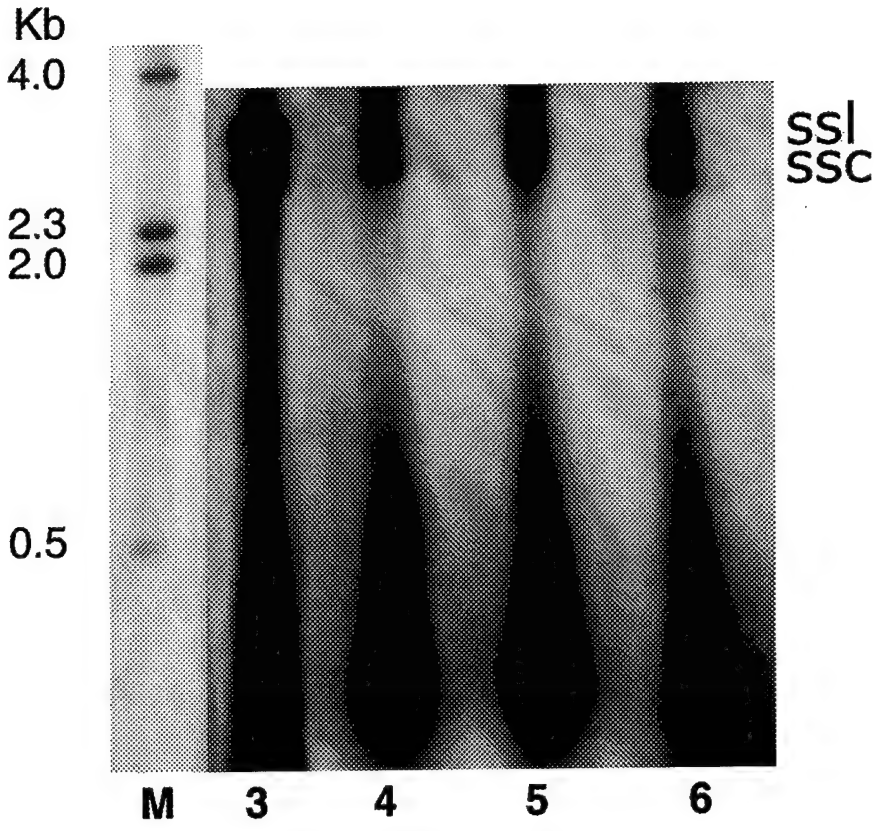


Figure 4. Neutral and alkaline agarose gel electrophoretic analyses of the DNA products synthesized by the DNA synthesome in the presence of SN-38, CPT and VP-16. *In vitro* DNA replication reactions (25 μl) containing the Q-Sepharose peak (15 μg), $pSVO^+$ DNA (50 ng), T-antigen and either DMSO (positive control), 0.2 μM SN-38, 0.5 μM CPT or 0.5 μM VP-16 were incubated for 1 hr at 37°C. The newly synthesized daughter DNA molecules were isolated and resolved by neutral or alkaline agarose gel electrophoresis (Materials and Methods). For neutral agarose gel electrophoresis, the position of form I, supercoiled DNA is indicated. Lane 1 shows that *Dpn*I-resistant form I, II and III DNAs as well as replicative and topological intermediates are produced by the DNA synthesome during *in vitro* replication reactions. Lane 2 shows that the synthesis of daughter DNA molecules by the DNA synthesome is T-antigen dependent. For alkaline agarose gel electrophoresis, the positions of double-stranded, covalently-closed circular and full-length, single-stranded linear DNA are shown. Size markers for the alkaline gels were derived from a *Hind* III digest of lambda DNA (New England Biolabs, Boston, MA) labeled with T4 DNA polymerase by standard methods (lane M) [34]. Lane 3 shows the DNA products synthesized *in vitro* by the DNA synthesome in the presence of DMSO (positive control); lanes 4-6 show the inhibitory effects of SN-38, CPT and VP-16, respectively, on DNA synthesome-mediated *in vitro* DNA replication.

the IC_{50} values for synthesome mediated DNA replication *in vitro*, the majority of the DNA molecules produced by the DNA synthesome ranged in size from only 100-700 nucleotides (Figure 4, lanes 4-6). These results indicate that the three agents strongly inhibited the elongation of nascent DNAs by the complex

DISCUSSION

In this report, we have presented for the first time data that indicate the breast cell DNA synthesome represents a novel model for studying the actions of SN-38 and VP-16. We found a close correlation between the IC_{50} values for the inhibition of intact cell and DNA synthesome-mediated *in vitro* DNA replication by CPT-11, or SN-38, (1.5 and 0.2 μM, respectively) and VP-16 (2.0 and 0.5 μM, respectively) (Table 1). These results further support the theory that the DNA synthesome represents the intact cell's DNA replication machinery. We also found that similar concentrations of SN-38 and VP-16, 0.5 μM each, inhibit DNA synthesome-associated topoisomerase I and II activities, respectively, by 50% (Figure 1, lane 2; Figure 2, lane 3); which is in agreement with the premise that SN-38 and VP-16 impair nucleic acid synthesis by selectively targeting topoisomerases I and II. Furthermore, it has been shown that both SN-38 and VP-16 inhibit the activities of topoisomerases I and II by stabilizing cleavable complexes that are normally short-lived intermediates in the catalytic cycles of the enzymes [10-13]. In accord with their modes of action, we found that the same concentrations of SN-38 and VP-16 that inhibit DNA synthesome-associated topoisomerase I and II activities by 50% (0.5 μM each), as well as inhibit DNA synthesome replication activity, also produce significant levels of cleavable complexes (Figure 3).

DNA topoisomerase I functions during the initiation and elongation stages of DNA synthesis as a swivelase, relieving superhelical stress in DNA caused by replication fork progression [16,38,40]. Although SN-38 inhibits the catalytic activity of topoisomerase I, this mechanism of action does not account for the agent's cytotoxicity, as topoisomerase

II can readily substitute for the enzyme [37]. Instead, a positive correlation exists between the efficacy of SN-38 as an anti-breast cancer agent and the amount of long-lived cleavable complexes produced by the agent in treated cells [41,42]. It has been postulated that the interaction of advancing replication forks with drug-stabilized cleavable complexes ultimately leads to cell death by causing replication fork breakage and the accumulation of irreparable DNA double strand breaks [13,16,43]. Several lines of evidence support this theory. First, arresting the progression of replication forks with aphidicolin, an inhibitor of DNA polymerases α and β, protects S-phase human leukemia (L1210) cells from CPT cytotoxicity without altering the cellular levels of cleavable complexes [44]. Second, Snapka et al. have reported that CPT adversely affects SV40 DNA synthesis in intact CV-1 cells by causing the rapid production of Cairns structures with broken DNA replication forks [16]. Third, it has been found that exposure of intact diploid human fibroblasts to CPT produces DNA strand breaks and inhibits DNA chain elongation in operating replicons [45]. Our observation that SN-38 causes an accumulation of short DNA molecules in *in vitro* replication reactions mediated by the DNA synthesome is consistent with these findings obtained in intact cells. Our result not only lends further support to the theory that drug stabilized topoisomerase I DNA cleavable complexes represent blocks to replication fork progression, but also supports the hypothesis that the DNA synthesome represents the intact cell's DNA replication machinery.

As described for SN-38, a positive correlation exists between the anti-cancer activity of VP-16 and the relative amount of cleavable complexes stabilized by the agent

[41,46]. Evidence for this relationship stems from the observation that wild-type but not VP-16 resistant MCF-7 human breast cancer cells show elevated levels of DNA strand breaks and decreased cell survival rates when exposed to increasing concentrations of the agent [47]. Conversely, VP-16 resistant cells of the human melanoma line FVP3 express normal levels of a functional topoisomerase II that is resistant to VP-16 stabilization of cleavable complexes [48]. The mechanisms by which the production of VP-16 stabilized cleavable complexes leads to cell death remain unresolved. Data from several studies indirectly suggest that the collision of moving replication forks with long-lived cleavable complexes is requisite for the cytotoxicity of topoisomerase II poisons. For example, cleavable complex forming topoisomerase II inhibitors demonstrate greater cytotoxicity against S phase cells as compared to cells in the G_1 or G_2/M phases of the cell cycle [49,50]. Also, aphidicolin pretreatment of lung fibroblasts synchronized in the S phase protects the cells from the lethal effects of topoisomerase II poisons without affecting the cellular levels of cleavable complexes [51]. Recently, Catapano et al. have provided strong evidence for the collision theory by demonstrating that the formation of VM-26 stabilized topoisomerase II DNA cleavable complexes within the first exon of the *c-myc* gene arrests replication fork progression during DNA synthesis in human leukemia (CEM) cells [52]. In the same study, Catapano et al. also found that VM-26 preferentially inhibits the elongation of nascent DNA molecules in intact CEM cells [52]. Our observation that VP-16 causes an accumulation of short DNA products during synthesome mediated *in vitro* replication is consistent with these results, lending additional support to the theory that

drug-stabilized topoisomerase II DNA cleavable complexes represent blocks to replication fork progression.

Although our results show a positive correlation between cleavable complex formation and the inhibitory effects of VP-16 on the replication activity of the DNA synthesome, it is possible that VP-16 impairs DNA synthesis and causes cell death by selectively targeting topoisomerase II catalytic activity. However, if this were true we would expect VP-16 to have blocked only the decatenation of mature daughter DNA molecules during the late stages of DNA synthesis mediated by the synthesome. In contrast, we observed that VP-16 caused an extensive accumulation of short DNA products during synthesome mediated replication *in vitro*. Nonetheless, we are currently performing experiments to examine the effects of pure topoisomerase II catalytic inhibitors on DNA synthesome replication activity.

Replication fork arrest by topoisomerase poisons may occur via several different mechanisms. For example, as both topoisomerases I and II are components of a multiprotein complex for DNA synthesis [24-31], formation of drug-stabilized topoisomerase DNA complexes may freeze the entire replication apparatus on template DNA, thereby impeding replication fork progression. Also, topoisomerase II concealed DNA cleavages formed in the presence of topoisomerase II poisons may be converted into DNA strand breaks by the action of a DNA helicase, as has been reported by others [52,53]. The resulting DNA strand breaks may halt DNA synthesis by causing the cellular replication machinery to run-off template DNA. In support of this claim, it has been shown that cleavage of substrate DNA by restriction endonucleases causes the replication

apparatus to run off template DNA in *in vitro* run-off replication reactions [54]. The DNA synthesome may serve as a useful model to study these potential consequences of cleavable complex formation.

In summary, our observation that SN-38 and VP-16 cause an accumulation of short DNA products during synthesome mediated *in vitro* DNA synthesis is consistent with the theory that long-lived topoisomerase DNA cleavable complexes represent blocks to replication fork progression. Furthermore, the latter result is especially significant, as the inhibition of the elongation of daughter DNA molecules by VP-16 has previously been reported for intact cells only [45,52]. We anticipate that future use of the breast cell DNA synthesome as a drug model will provide important insights into the mechanisms of action of SN-38 and VP-16.

REFERENCES

1. Landis, S.H., Murray, T., Bolden, S. and Wingo, P.A. (1998) Cancer statistics, 1998. CA: Cancer J. for Clin. **48**: 6-29.
2. Bonadonna, G., Valagussa, B.S., Zucali, R. and Salvadori, B. (1995) Primary chemotherapy in surgically resectable breast cancer. CA Cancer J Clin. **45**: 227-243.
3. Anonymous. (1992) Systemic treatment of early breast cancer by hormonal, cytotoxic or immune therapy. Lancet **339**: 1-15.
4. Anonymous. (1992) Systemic treatment of early breast cancer by hormonal, cytotoxic or immune therapy. Lancet **339**: 71-85.
5. Hortobagyi, G.N., Buzdar, A.U., Strom, E.A., Ames, F.C. and Singletary, S.E. (1995) Primary chemotherapy for early and advanced breast cancer. Cancer Lett. **90**: 103-109.
6. Boyd, M.R. (1993) The future of new drug development. In: *Current Therapy in Oncology*. B.C. Decker, Inc., pp. 11-22.
7. Abrams, J.S., Moore, T.D. and Friedman, M. (1994) New chemotherapeutic agents for breast cancer. Cancer (Suppl.) **74**: 1164-1176.
8. Terret, C., Couteau, C. and Armand, J.P. (1996) CPT-11: the European clinical development. J Infusional Chemother. **6**: 152-157.
9. Thomas, G.W., Muss, H.B., Jackson, D.V., McCulloch, J., Ramseur, W., McFarland, J., Hoen, H., Pavy, M. and Heath, R. (1994) Vincristine with high dose etoposide in advanced breast cancer: a phase II trial of the Piedmont Oncology Association. **CCP** 35: 165-168.

10. Osheroff, N., Corbett, A. and Robinson, M.J. (1994) Mechanism of action of topoisomerase II-targeted antineoplastic drugs. Adv. Pharmacol. **29B**: 105-124.

11. Pommier, Y., Tanizawa, A. and Kohn, K.W. (1994) Mechanisms of topoisomerase I inhibition by anticancer agents. Adv. Pharmacol. **29B**: 73-92.

12. Creemers, G.J., Lund, B. and Verweij, J. (1994) Topoisomerase I inhibitors: topotecan and irinotecan. Cancer Treat. Rev. **20**: 73-96.

13. Liu, L.F. (1989) DNA topoisomerase poisons as antitumor drugs. Annu. Rev. Biochem. **58**: 351-375.

14. D'Arpa, P. and Liu, L.F. (1989) Topoisomerase-targeting antitumor drugs. Biochim. Biophys. Acta **989**: 163-177.

15. Tsao, Y-P, Russo, A., Nyamuswa, G., Silber, R. and Liu, L.F. (1993) Interaction between replication forks and topoisomerase I-DNA cleavable complexes: studies in a cell-free SV40 DNA replication system. Cancer Res. **53**:5908-5914.

16. Snapka, R.M. (1986) Topoisomerase inhibitors can selectively interfere with different stages of simian virus 40 DNA replication. Mol. Cell. Biol. **6**: 4221-4227.

17. Ishimi, Y., Sugasawa, K., Hanaoaka, F., Eki, T. and Hurwitz, J. (1992) Topoisomerase II plays an essential role as a swivelase in the late stage of SV40 chromosome replication *in vitro*. JBC **267**: 462-466.

18. Ishimi, Y., Ishida, R.J. and Andoh, T. (1992) Effect of ICRF-193, a novel DNA topoisomerase II inhibitor, on simian virus 40 DNA and chromosome replication *in vitro*. Mol. Cell. Biol. **12**: 4007-4014.

19. Hsiang, Y.H., Hertzberg, R., Hecht, S. and Liu, L.F. (1985) Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J. Biol. Chem. **260**: 14873-14878.

20. Riou, J-F, Fosse, P., Nguyen, C.H., Larsen, A.K., Bissery, M-C, Grondard, L., Saucier, J-M, Bisagni, E. and Lavelle, F. (1993) Intoplicine (RP 60475) and its derivatives, a new class of antitumor agents inhibiting both topoisomerase I and II activities. Cancer Res. **53**: 5987-5993.

21. Kufe, D.W. and Major, P.P. (1982) Studies on the mechanism of action of cytosine arabinoside. Med Pediatr. Oncol. Suppl. **1**: 49-67.

22. Glisson, B., Gupta, R., Smallwood-Kentro, S. and Ross, W. (1986) Characterization of acquired epipodophyllotoxin resistance in a chinese hamster ovary cell line: loss of drug-stimulated DNA cleavage activity. Cancer Res. **46**: 1934-1938.

23. Yang, L., Wold, M.S., Li, J.L., Kelly, T.J. and Liu, L.F. (1987) Roles of DNA topoisomerases in simian virus 40 DNA replication *in vitro*. Proc. Natl. Acad. Sciences **84**: 950-954.

24. Hickey, R.J., Malkas, L.H., Pedersen, N., Li, C. and Baril, E.F. (1988) Multienzyme complex for DNA replication in HeLa cells. Moses, R. and Summers, W. (eds.) In: *DNA Replication and Mutagenesis*, Amer. Soc. Microbiol. Public., Washington D.C., pp. 41-54.

25. Malkas, L.H., Hickey, R.J., Li, C.J., Pedersen, N. and Baril, E.F. (1990) A 21S enzyme complex from HeLa cells that functions in simian virus 40 (SV40) DNA replication *in vitro*. Biochem. **29**: 6362-6374.

26. Applegren, N., Hickey, R.J., Kleinschmidt, A.M., Zhou, Q., Coll, J., Wills, P., Swaby, R., Wei, Y., Quan, J.Y., Lee, M. and Malkas, L.H. (1995) Further characterization of the human cell multiprotein DNA replication complex (MRC). J. Cell. Biochem. **59**: 91-107.

27. Wu, Y., Hickey, R.J., Lawlor, K., Wills. P., Yu, F., Ozer, H., Starr, R., Quan, J.Y., Lee, M. and Malkas, L.H. (1994) A 17S multiprotein form of murine cell DNA polymerase mediates polyoma virus DNA replication *in vitro*. J. Cell. Biochem. **54**: 32-46.

28. Lin, S., Hickey, R.J. and Malkas, LH. (1997) The isolation of a DNA synthesome from human leukemia cells. Leuk. Res. **21**: 501-512.

29. Coll, J.M., Weeks, J.G., Hickey, R.J., Schnaper, L., Yue, W., Brodie, A.M.H., Syvaoja, J. and Malkas, L.H. (1996) The human breast cell DNA synthesome: Its purification from tumor tissue and cell culture. Oncol. Res. **8**: 435-447.

30. Tom, T., Malkas, LH. and Hickey, R.J. (1995) Identification of multiprotein complexes containing DNA replication factors by native immunoblotting of HeLa cell protein preparations with T-antigen dependent SV40 DNA replication activity. J. Cell. Biochem. **63**: 259-267.

31. Coll, J.M., Hickey, R.J., Cronkey, E.A., Jiang, H-Y, Schnaper, L., Lee, M.Y.W.T., Uitto, L., Syvaoja, J.E. and Malkas, L.H. (1997) Mapping specific protein-protein interactions within the core component of the breast cell DNA synthesome. Oncol. Res. **9**: 629-639.

32. Hickey, R.J. and Malkas, L.H. (1997) Mammalian cell DNA replication. In: *Critical Reviews in Eukaryotic Gene Expression*, **7**: 125-157.

33. DePamphilis, M.L. and Bradley, M.K. (1986) Replication of SV40 and Polyoma Virus Chromosomes. Salzman, N.P. (ed.) In: *The Papovaviridae Volume I: The Polyomavirus*, pp. 99-246, Plenum Press, New York, NY.

34. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) In: Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. E.1-E.39.

35. Stillman, B., Gerard, R.D., Guggenheimer, R.A. and Gluzman, Y. (1985) T-antigen and template requirements for SV40 DNA replication *in vitro*. EMBO J. **4**: 2933-2939.

36. Kawato, Y., Aonuma, M., Hirota, Y., Kuga, H. and Sato, K. (1991) Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. Cancer Res. **51**: 4187-4191.

37. Tadou, G., Portemer, C., Jaxel, C. and Duguet, M. (1993) Inhibition of DNA synthesis and DNA fragmentation in stimulated splenocytes by the concerted action of topoisomerase I and II poisons. Biochem. Pharmacol. **45**: 331-337.

38. Coll, J., Hickey, R.J., Wei, Y. and Malkas, L.H. (1996) The human cell multiprotein DNA replication complex (MRC): The effect of camptothecin on its ability to support *in vitro* DNA synthesis. Cancer Chemother. Pharmacol. **39**:97-102.

39. Hsiang, Y.H. and Liu, L.F. (1988) Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. Cancer Res. **48**: 1722-1726.

40. Dean, F.B., Bullock, P., Murakami, Y., Wobbe, C.R., Weissback, L. and Hurwitz, J. (1987) Simian virus 40 (SV40) DNA replication *in vitro*: SV40 large T-antigen unwinds DNA containing the SV40 origin of replication. Proc. Natl. Acad. Sciences **84**: 16-20.

41. Boege, F. (1996) Analysis of Eukaryotic DNA topoisomerases and topoisomerase-directed drug effects. Eur. J. Clin. Chem. Clin. Biochem. **34**: 873-888.

42. Tsao, Y-P, D'Arpa, P. and Liu, L.F. (1992) The involvement of active DNA synthesis in camptothecin-induced G_2 arrest: altered regulation of $p34^{cdc2}$/cyclin B. Cancer Res. **52**: 1823-1829.

43. Avemann, K., Knippers, R., Koller, T. and Sogo, J.M. (1988) Camptothecin, a specific inhibitor of type I DNA topoisomerase, induces DNA breakage at replication forks. Mol. Cell. Biol. **8**: 3026-3034.

44. Hsiang, Y-H, Lihou, M.G. and Liu, L.F. (1989) Arrest of replication forks by drug-stabilized topoisomerase I cleavable complexes as a mechanism of cell-killing by camptothecin. Cancer Res. **49**: 5077-5082.

45. Kauffman, W.K., Boyer, J.C., Estabrooks, L.L. and Wilson, S.J. (1991) Inhibition of replicon initiation in human cells following stabilization of topoisomerase-DNA cleavable complexes. Mol. Cell. Biol. **11**:3711-3718.

46. Ross, W.E., Rowe, T., Glisson, ., Yalowich, J. and Liu, L. (1984) Role of

topoisomerase II in mediating epipodophyllotoxin-induced DNA cleavage. Cancer Res. **44**: 5857-5860.

47. Sinha, B.K., Haim, N., Dusre, L., Kerrigan, D. and Pommier, Y. (1988) DNA strand breaks produced by etoposide (VP-16, 213) in sensitive and resistant human breast tumor cells: implications for the mechanisms of action. Cancer Res. **48** (18): 5096-5100.
48. Campain, J.A., Padmanabhan, R., Hwang, J., Gottesman, M.M. and Pastan, I. (1993) Characterization of an unusual mutant of human melanoma cells resistant to anticancer drugs that inhibit topoisomerase II. J Cell. Physiol. **155**: 414-425.
49. Chow, K-C and Ross, W.E. (1987) Topoisomerase-specific drug sensitivity in relation to cell cycle progression. Molec. Cell. Biol. **7**: 3119-3123.
50. D'Arpa, P., Beardmore, C. and Liu, L.F. (1990) Involvement of nucleic acid synthesis in cell killing mechanisms of topoisomerase poisons. Cancer Res. **50**: 6919-1924.
51. Holm, C., Covey, J.M., Kerrigan, D. and Pommier, Y. (1989) Differential requirement of DNA replication for the cytotoxicity of DNA topoisomerase I and II inhibitors in Chinese hamster DC3F cells. Cancer Res. **49**: 6365-6368.
52. Catapano, C.V., Carbone, G.M.R., Pisani, F., Qiu, J. and Fernandes, D.J. (1997) Arrest of replication fork progression at sites of topoisomerase II-mediated DNA cleavage in human leukemia CEM cells incubated with VM-26. Biochem. **36**: 5739-5748.
53. Howard, M.T., Neece, S.H., Matson, S.W. and Kreuzer, K.N. (1994) Disruption of a topoisomerase-DNA cleavage complex by a DNA helicase. Proc. Natl. Acad. Sci.

U.S.A. **91**: 12031-12035.

54. Leffak, M. and James, C.D. (1989) Opposite replication polarity of the germ line c-*myc* gene in HeLa cells compared with that of two Burkitt lymphoma cell lines. Molec. Cell. Biol. **9**: 586-593.

Chapter 5. Conclusion and Future Aims

From the work presented in this thesis, we have drawn several important conclusions about breast cell DNA replication. Foremost, we have demonstrated that breast cells possess a multiprotein complex of replication-essential polypeptides, designated the DNA synthesome, that is competent to fully support SV40 DNA replication *in vitro*. Second, we have found that the core component of the breast cell DNA synthesome represents a complex of tightly associated DNA synthetic elongation factors. Evidence for this stems from our observation that DNA polymerases α, δ, DNA primase, PCNA and RF-C each coprecipitates with antibodies directed against DNA polymerases α, δ and PCNA. Third, we have found that the DNA synthesome isolated from breast cancer cells and tissue has a comparatively lower fidelity for DNA replication than the synthesome isolated from a normal breast cell line. This discrepancy in replication fidelity between the malignant and non-malignant breast cell DNA synthesome suggests that transformation to the malignant phenotype alters the process by which the synthesome from normal cells replicates DNA. Finally, utilizing the breast cell DNA synthesome as a model for studying the mechanisms of action of SN-38 and VP-16, we obtained compelling data that suggests both drug-stabilized topoisomerase I and II cleavable complexes represent blocks to replication fork progression.

One of our future research objectives is to generate a three-dimensional map of all the protein-protein interactions within the breast cell DNA synthesome. In order to accomplish this goal, it will be necessary to determine where those components that did not coprecipitate with antibodies directed against PCNA or DNA polymerases α and δ (i.e., DNA ligase I) lay in reference to the core complex. Moreover, to construct a

three-dimensional map every protein component of the DNA synthesome must be identified and the subunit composition and stoichiometry of each component determined. Recent work in our laboratory indicates that in addition to DNA replication proteins, several DNA repair proteins copurify with the breast cell DNA synthesome [1]. These factors include the following mismatch repair proteins: hMSH2, hMSH6, hMLH1, hPMS1, hPMS2, Kw80 and MYH. Furthermore, the DNA synthesome is capable of specifically recognizing and binding to single DNA mismatches and insertion-deletion loops of two and four nucleotides [1]. That DNA repair polypeptides serve as components of the breast cell DNA synthesome is consistent with the results of numerous studies demonstrating an intimate link between the processes of DNA replication and repair [2-4]. For example, RP-A, DNA polymerase ε, RF-C, PCNA and DNA ligase I, which are proteins that play integral roles in mammalian cell DNA synthesis [reviewed in 5; 6], cooperate with approximately eighteen other polypeptides in *in vitro* nucleotide excision reactions reconstituted with purified components [2]. Also, it has been shown that PCNA associates with MLH1 and MSH2 in yeast, and is required for efficient mismatch repair [3]. Determining the physical organization of the DNA repair proteins within the breast cell DNA synthesome is one of our next immediate tasks for the generation of an accurate map of the protein-protein interactions within the breast cell DNA synthesome.

A second future research objective for our laboratory is to elucidate the mechanisms by which the DNA replication fidelity and repair activity of the breast cell DNA synthesome are regulated. We postulate that disruption of these processes during the course of cellular transformation may potentiate the increased rate of genetic mutation

and genomic instability observed in breast cancer cells. Indeed, such a mutator hypothesis has been described for hereditary nonpolyposis colorectal cancer (HNPCC), in which error-prone DNA synthesis and mutations in DNA repair proteins of tumor cells cause an accumulation of extensive cellular DNA damage [7]. As described in a previous chapter of this thesis, the DNA synthesome isolated from human breast cancer cells and tissue possesses a replication fidelity approximately 5-6 fold lower than that of the synthesome isolated from a non-malignant human breast cell line. In a recent report from our laboratory, Sekowski et al. further examined these discrepancies in replication fidelity between the malignant and non-malignant breast cell DNA synthesome. It was found that the decreased replication fidelity of the malignant breast cell DNA synthesome derived from cell lines (MCF 7, MDA MB-468 and Hs578T) or human tumor tissue does not merely result from an increased *in vitro* replication activity of the complex or a hyperplastic pathology of breast tumor tissue. Furthermore, a correlation was found between the decreased replication fidelity of the malignant breast cell DNA synthesome and an accumulation of mutations (insertions, deletions and mismatches) within the replication products created by the complex. These results demonstrate for the first time that functional alterations of the DNA synthetic and/or repair machinery of human breast cancer cells lead to the production of genetic mutations.

In a related report from our laboratory, Bechtel et al. provide strong evidence for a relationship between the decreased replication fidelity of the malignant breast cell DNA synthesome and the presence of a novel form of PCNA within the complex [9]. Utilizing two-dimensional polyacrylamide gel electrophoretic analysis (2D PAGE), the DNA synthesome isolated from several malignant breast cell lines (MCF 7, MDA MB-468 and

Hs578T) as well as from human lobular and ductal breast cancer tissues was shown to possess a form of PCNA that migrates with an acidic isoelectric point (pI). In contrast, a basic form of PCNA was found in the DNA synthesome purified from non-malignant breast (MCF 10A) cells and tissue. As the nucleotide sequences of clones encoding the PCNA genes derived from malignant and non-malignant breast cell lines were identical, the novel form of PCNA most likely results from an altered post-translational modification. In support of this, Simbulan et al. have shown that PCNA is a target for poly(ADP) ribosylation [10]. Additionally, phosphorylation may represent a possible modification as it has been reported that PCNA is weakly phosphate-labeled and coprecipitates with a number of associated protein kinases [11]. Experiments are currently underway in our laboratory to determine the type of post-translational modification made to PCNA in breast cancer cells. Furthermore, the effects of such an alteration on the ability of PCNA to interact with DNA replication and repair proteins will also be examined.

In both of the aforementioned studies from our laboratory, a decreased fidelity for DNA replication by the synthesome and the novel form of PCNA were found in 100% of the malignant breast cells examined [8,9]. These results are significant because they indicate that both a decreased replication fidelity and the novel form of PCNA represent hallmarks of malignancy in breast cells. Consequently, each may serve as a better indicator of breast cell malignancy than other currently used molecular markers (i.e., p53, her2/neu, progesterone and estrogen receptor status) which are found in only a fraction of all breast tumors. Efforts are presently being made to develop the decreased replication

fidelity of the synthesome and the novel form of PCNA as markers for the detection and diagnosis of breast cancer.

A third future research objective for our laboratory, stemming from the work presented in this thesis, is to employ the DNA synthesome as a model to study in detail the modes of action of SN-38 and VP-16. A number of studies performed in intact cells have demonstrated that SN-38 and VP-16-stabilized topoisomerase DNA cleavable complexes represent blocks to replication fork progression [12-14]. Our observation that these agents cause an accumulation of short DNA molecules by the DNA synthesome during replication *in vitro* are consistent with these reports. However, the actual mechanisms by which SN-38 and VP-16-stabilized cleavable complexes impede replication fork progression are currently unknown. It has been postulated that either component of long-lived cleavable complexes, the covalent topoisomerase-DNA complexes or DNA strand breaks, can impede the translocation of replication machinery along template DNA [14]. As both topoisomerases I and II serve as components of a multiprotein complex, it is also possible that topoisomerase poisons block the movement of the replication machinery along DNA by freezing the entire complex on template DNA. We are currently performing experiments to address these hypotheses.

Finally, several lines of evidence indicate that SN-38 inhibits topoisomerase I activity during the initiation and elongation stages of DNA synthesis [15,16]. There are several conflicting reports, however, regarding at which stage of DNA synthesis VP-16 poisons topoisomerase II activity. For example, Snapka et al. found that VP-16 acts during the late stages of DNA synthesis in CV-1 cells to block the decatenation of intertwined daughter DNA molecules [12,17]. In contrast, Kauffman et al. observed that

the exposure of intact diploid human fibroblasts to VM-26, a topoisomerase II inhibitor with a mechanism of action similar to that of VP-16, inhibits DNA chain elongation in operating replicons [18]. Our observation that VP-16 caused an extensive accumulation of short daughter DNA molecules during synthesome mediated *in vitro* replication is consistent with the latter result and may be interpreted to suggest that topoisomerase II normally functions during the elongation stage of DNA synthesis. More work is necessary to confirm this role for topoisomerase II during DNA synthesis. We anticipate that future work with the breast cell DNA synthesome will provide important insights into the mechanisms of action of SN-38 and VP-16 as well as the roles of topoisomerases in breast cell DNA replication.

References

1. Sekowski et al., unpublished data (1998).

2. Aboussekhra; A.; Biggerstaff, M.; Shivji, M.K.K.; Vilpo, J.A.; Moncollin, V.; Podust, V.N.; Protic, M.; Hubscher, U.; Egly, J-M; Wood, R.D. Mammalian DNA nucleotide excision repair reconstituted with purified protein components. Cell **80**:859-868; 1995.

3. Shivji, M.K.K; Podust, V.N.; Hubscher, U.; Wood, R.D. Nucleotide excision repair DNA synthesis by DNA polymerase ε in the presence of PCNA, RF-C and RP-A. Biochem. **34**: 5013-5017; 1995.

4. Foiani, M.; Lucchini, G.; Plevani, P. The DNA polymerase α-primase complex couples DNA replication, cell-cycle progression and DNA damage response. TIBS **22**: 424-427; 1997.

5. Hickey, R.J. and Malkas, L.H. Mammalian cell DNA replication. In: *Critical Reviews in Eukaryotic Gene Expression* **7**: 125-157; 1997.

6. Tuusa, J.; Uitto, L.; Syvaoja, J. Human DNA polymerase ε is expressed during cell proliferation in a manner characteristic of replicative DNA polymerases. Nucl. Acids Res. **23**: 2178-2183; 1995.

7. Weinberg, R.A.; Hanahan, D. The molecular pathogenesis of cancer. In: *Molecular Oncology*. Scientific American Inc., New York; 1996: 179-204.

8. Sekowski, J.W.; Malkas, L.H.; Schnaper, L.; Bechtel, P.; Long, B.J.; Hickey, R.J. Human breast cancer cells contain an error-prone DNA replication apparatus. Cancer Res. (in press); 1998.

9. Bechtel, P.E.; Hickey, R.J.; Schnaper, L.; Sekowski, J.W.; Long, B.J.; Freund, R.; Liu, N.; Rodriguez-Valenzuela, C.; Malkas, L.H. A unique form of proliferating cell nuclear antigen (PCNA) is present in malignant breast cells. Cancer Res. (in press); 1998.

10. Simbulan, C.M.G.; Rosenthal, D.S.; Hilz, H.; Hickey, R.J.; Malkas, L.H.; Applegren, N.; Wu, Y.; Bers, G.; Smulson, M.E. The expression of poly(ADP-ribose) polymerase during differentiation linked DNA replication reveals that it is a component of the multiprotein DNA replication complex. Biochem. **35**:11622-33; 1996.

11. Brand, S.R.; Bernstein, R.M.; Mathews, M.B.; Trimeric structure of human proliferating cell nuclear antigen. Implications for enzymatic function and autoantibody recognition. J. Immunol. **153**: 3070-3079; 1994.

12. Snapka, R.M. Topoisomerase inhibitors can selectively interfere with different stages of simian virus 40 DNA replication. Mol. Cell. Biol. **6**: 4221-4227; 1986.

13. Avemann, K.; Knippers, R.; Koller, T.; Sogo, J.M. Camptothecin, a specific inhibitor of type I DNA topoisomerase, induces DNA breakage at replication forks. Mol. Cell. Biol. **8**: 3026-3034; 1988.

14. Catapano, C.V.; Carbone, G.M.R.; Pisani, F.; Qiu, J.; Fernandes, D.J. Arrest of replication fork progression at sites of topoisomerase II-mediated DNA cleavage in human leukemia CEM cells incubated with VM-26. Biochem. **36**: 5739-5748; 1997.

15. Waga, S.; Bauer, G.; Stillman, B. Reconstitution of complete SV40 DNA replication with purified replication factors. JBC **269**: 10923-10934; 1994.

16. Shin, C.G.; Snapka, R.M. Patterns of strongly protein-associated simian virus 40 DNA replication intermediates resulting from exposure to specific topoisomerase poisons. Biochem. 29: 10934-10939; 1990.

17. Snapka, R.M.; Permana, P.A. SV40 DNA replication intermediates: analysis of drugs which target mammalian DNA replication. Bioessays **15**: 121-127; 1993.

18. Kauffman, W.K.; Boyer, J.C.; Estabrooks, L.L.; Wilson, S.J. Inhibition of replicon initiation in human cells following stabilization of topoisomerase-DNA cleavable complexes. Mol. Cell. Biol. **11**:3711-3718; 1991.

revised July 14, 1998

Curriculum Vitae

Jennifer M. Coll

4809 Roland Avenue
Apartment 3A
Baltimore, MD 21210
jcoll@umabnet.ab.umd.edu

Education

University of Maryland at Baltimore, *Class of 2001*
School of Medicine
Baltimore, Maryland
Medical student

University of Maryland at Baltimore *1993-1998*
Baltimore, Maryland
PhD candidate, Department of Pharmacology and Experimental Therapeutics

University of Notre Dame *1988-1992*
Notre Dame, Indiana
B.S., Biological sciences

Research Experience

Doctoral Research
Characterization of a multiprotein complex for DNA replication, designated the DNA synthesome, from human breast cancer cells and breast tumor tissue. Examining the mechanisms of action of the DNA topoisomerase I and II inhibitors, camptothecin and etoposide, respectively, on DNA synthesome mediated *in vitro* DNA replication.

Research Interests

- Mechanisms and regulation of mammalian cell DNA replication
- Hormonal regulation of cellular proliferation

Awards and Recognition

- United States Army Medical Research and Development Command Breast Cancer Predoctoral Fellowship, 1994-present
- Who's Who Among U.S. Graduate Students, 1996
- The National Dean's List, 1996
- University of Maryland Cancer Center Travel Award, 1996
- Invited Participant to the American Association for Cancer Research sponsored *Histopathobiology of Cancer Workshop*, Keystone, CO, July, 1995
- University of Maryland Graduate Student Travel Award, 1995
- Sigma Xi Scientific Society Grant-in-Aid of Research, 1994

Professional Memberships

- Associate Member of the American Association for Cancer Research, 1995-present
- Sigma Xi, The Scientific Research Society, 1996-present
- Federation of American Societies for Experimental Biology (FASEB), 1996-present
- Women in Cancer Research, 1996-present
- Association for Women in Science, 1994-present
- Breast Cancer Coalition, 1996
- Department of Pharmacology and Experimental Therapeutics representative to the University of Maryland Graduate Student Government Association, 1993

Mentoring Experience

- Research advisor to Erica A. Cronkey, Masters student, Department of Pharmacology and Experimental Therapeutics, UMAB
- Research advisor to Rosemin Daya, Medical and Technology Research student, UMAB

Inventions and Patents

- Mini-fractionation scheme for the purification of the DNA synthesome from tissue samples, 1997 (UMAB disclosure # pending)

Abstracts

- **J. Coll**, E. Cronkey, R.J. Hickey, L. Schnaper and L.H. Malkas, The human breast cell DNA synthesome: the effects of irenotecan and etoposide on its ability to support *in vitro* DNA synthesis. Presented at the Annual 1997 Meeting of the American Association for Cancer Research held in San Diego, CA.

- C. Simbulan-Rosenthal, D. Rosenthal, R.J. Hickey, L.H. Malkas, **J. Coll** and M. Smulson, Poly(ADP-ribose) polymerase (PARP) is required for expression or assembly of components of the multiprotein DNA replication complex (MRC). Presented at the 1997 Annual Meeting of the American Society for Biochemistry and Molecular Biology.

- **J. Coll**, E.A. Cronkey, R.J. Hickey, L. Schnaper and L.H. Malkas, The breast cell DNA synthesome: a novel model for examining the mechanisms of action of irenotecan and etoposide. Presented at the 1997 U.S. Army Medical Research and Development Command Era of Hope Breast Cancer Research Meeting, Washington, D.C.

- **J. Coll,** R.J. Hickey, L. Schnaper, Y. Wei and L.H. Malkas, Protein-Protein interactions within the human breast cell DNA synthesome. Presented at the 1996 Annual San Antonio Breast Cancer Symposium held in San Antonio, Texas.

- **J. Coll**, R.J. Hickey, Y. Wei and L.H. Malkas, The multiprotein DNA replication complex (MRC): The effect of camptothecin on its ability to support DNA synthesis in vitro. Presented at the 1996 New York Academy of Sciences Meeting on Camptothecins held in Bethesda, MD.

- **J. Coll**, R.J. Hickey, J. Weeks-Sekowski, L. Schnaper, W. Yue, A.H.M. Brodie and L.H. Malkas, Human breast cancer cells mediated DNA replication via an organized DNA synthesome. Presented at the 1996 Annual Meeting of the American Association for Cancer Research held in Washington, D.C.

• P. Bechtel, **J. Coll**, L. Malkas and Hicley, R.J., The identification of structural alterations in the DNA synthetic apparatus of human breast cancer cells. Presented at the 1996 Annual Meeting of the American Association for Cancer Research held in Washington, D.C.

• **J. Coll**, Y. Wei, R.J. Hickey and L.H. Malkas, Protein-Protein interactions within the human breast cell DNA synthesome. Presented at the 1996 McGill University Conference on the Regulation of Eukaryotic DNA Replication held in Quebec, Canada.

• **J. Coll**, Y. Wei and L.H. Malkas, A unique model to investigate the mechanisms of action of camptothecin on DNA synthesis. Presented at the 1994 American Society of Biochemistry and Molecular Biology Meeting held in Washington, D.C.

• L. Malkas, Y. Wu, N. Applegren, N. Li, **J. Coll**, N. Tuteja and R.J. Hickey, The mammalian cell multiprotein DNA replication complex (MRC). Presented at the 1994 American Society of Biochemistry and Molecular Biology Meeting held in Washington, D.C.

• **J. Coll**, J. Glazer, R.J. Hickey and L.H. Malkas, Multiprotein replication complex protein-protein interactions. Presented at the 1994 McGill University Conference on the Regulation of Eukaryotic DNA Replication held in Montreal, Quebec, Canada.

Platform Presentations

• Protein-Protein interactions within the breast cancer cell DNA synthesome **(J. Coll)**. Presented at the 1997 Annual Graduate Student Research Day, UMAB.

Selected Publications

• **J.M. Coll**, E.A. Cronkey, Hickey, R.J. and Malkas, L.H. (1998) The DNA Synthesome: A Novel Model for Examining the Mechanisms of Action of Irinotecan and Etoposide. Biochem. Pharmacol. (submitted).

• **J.M. Coll**, R.J. Hickey, E.A. Cronkey, H.Y. Jiang, L.Schnaper, L. Uitto, J.E. Syvaoja and L.H. Malkas (1997). Mapping Specific Protein-Protein Interactions within the Core Component of the Breast Cell DNA Synthesome. Oncology Res. 9: 629-639.

• **J.M. Coll**, J.W. Sekowski, R.J. Hickey, L. Schnaper, W. Yue, A.M.H. Brodie, L. Uitto, J.E. Syvaoja and L.H. Malkas (1996). The Human Breast Cell DNA Synthesome: Its Purification from Tumor Tissue and Cell Culture. Oncology Res. 8: 435-447.

•N. Applegren, R.J. Hickey, A.M. Kleinschmidt, Q. Zhou, **J.M. Coll**, P. Wills, R. Swaby, Y. Wei, J.Y. Quan, M.Y.W.T. Lee and L.H. Malkas (1995). Further Characterization of the Human Cell Multiprotein DNA Replication Complex. J. Cell. Biochem. 59: 91-107.